CELLULAR DIFFERENTIATION IN BONE

Discussion Leader:

DR. JOHN J. PRITCHARD

PRITCHARD: Ladies and gentlemen, we shall be talking this morning about "Cellular Differentiation in Bone." The first difficulty is the lack of precision in this word "differentiation." There are several meanings, and quite possibly we shall be thinking about differentiation in different ways at different times. You may have other meanings that I have not thought of, but perhaps for the moment I may be allowed to tell you how I think the word "differentiation" can be parsed and analyzed.

First, the word "differentiation" can apply, and often is applied, to a population of cells. Secondly, the word "differentiation" can apply to some individual cell within a population. It depends on whether our attention is focused on the whole population or on one particular element in that population.

Then again, we can focus on static or instantaneous aspects of differentiation, the idea of differentiation being, like beauty, in the eye of the beholder. The beholder sees a picture; he sees it made up of differing elements and tries to differentiate, delineate, and clarify the elements into species, to which he gives names. This is purely a static analysis; time does not enter into it. You could all die suddenly, but you could still be differentiated by your appearances and classified into groups, and that is normally what the histologist does. He may think he is doing something more dynamic, but really he is analyzing a dead, fixed picture.

The "static" man is simply concerned with differences within a population at a given point in time. The dynamic aspect of differentiation is concerned with change, but even here there is more than one meaning behind the idea of change. There is the idea of the homogeneous becoming heterogeneous, with differences arising and increasing as in the development of an embryo. There is also the idea of the transformation of one mode into another mode without it necessarily being part of an ontologic process.

In these transformations there are those we consider irreversible, sometimes called true differentiation, and those changes which we consider to be reversible, often referred to as "modulation."

Under the heading of "differentiation," therefore, we have population studies, individual cell studies, static studies, dynamic studies. Cellular differentiation in bone thus implies at least four kinds of activity.

FREMONT-SMITH: "Irreversible" means not yet known to be reversible.

PRITCHARD: That is true. It also means that the change is carried on to the next generation of cells.

FREMONT-SMITH: The inheritance of an acquired characteristic.

PRITCHARD: Yes; from one cell to another, not from one organism to another. Some of you may want to add to this analysis of what we mean by "differentiation." In the meantime, I would like to add one or two more guidelines to the discussion. Suppose we begin with the concept of a bone-cell population. These populations are not the same everywhere. There is a great variation in the local populations in different parts of a bone, in different bones, at different ages, in different species, in different functional states. So, we have variations in populations.

There are also variations within a population. This takes into account the heterogeneity within a given population. We are looking eventually for differences between cells. We have to be careful about the criteria we use for differentiating cells, and we have to spend a little time, I think, on nomenclature. What are we going to call those cells once we differentiate them out of the population?

These questions belong to the static level of histologic study. Dynamic studies may be summed up as activities of the population of bone cells. This covers a multitude of topics, and we obviously cannot discuss them all. However, I want to give a fairly complete frame of reference and then select some of the more important issues; you can introduce other important ones.

Under activities of a population, we must not forget the movements of cells, which are sometimes ignored. There are respiratory and secretory activities. There is mitosis, and death of cells. There is the recruitment of cells to a population, and there is the emigration of cells from a population. Finally there is differentiation.

FREMONT-SMITH: You start with a population of bone cells. How did they get that way?

PRITCHARD: You mean, do we get cells from outside?

FREMONT-SMITH: There is an ontogeny of these cells——

PRITCHARD: An ontogeny which consists of mitosis followed by migration, differentiation, modulation, transformation, functional activity; in other words, the population has a lively history which can

be traced back to embryonic beginnings at an ossification center, and perhaps even earlier. Moreover, these activities are under control.

In principle, I think there are three kinds of control that can be exerted over the activities of the population. First, there is control from outside the population, directly issuing from sources external or extrinsic to the population. One thinks of hormones, mechanical factors, toxic substances—anything that can get at a population from outside.

FREMONT-SMITH: Do you consider induction as part of that?

PRITCHARD: Induction is sometimes controlled from outside, sometimes from inside.

FREMONT-SMITH: You are starting as if we already had a population of bone cells.

PRITCHARD: We will go back and prove that there is a population later on. But assume that there is a large population of cells and that these cells are active in some of the ways I have mentioned; then we could have influences on the activity of a population arising from outside the population. We could also have mutual influences of the cells upon one another within the population.

We also have influences or determinants which come from the cell itself, the DNA in one cell perhaps being in a different state from the DNA in another cell. Therefore, there are controls extrinsic to the population and controls intrinsic to the cell.

Then, there are some final considerations: How do these controls work? Do controls affect the population by affecting the mitotic rate of some particular stem cell? Do they affect the secretory activity of some already modulated cell? Do they influence membrane functions in some of the cells? Or do these controls work at the DNA–RNA synthesis level? Just what kinds of controls and regulations are present, and how do they work?

These are my four major headings: the static attributes of the population, the activities of the population, the controls, and the mechanisms of control. Some key problems center around the criteria for identifying cell types and giving them names. The most important problem is tracing individual cell lineages through serial mitoses and isolation.

The most pressing problems, however, concern the controlling mechanisms—and we tend to think immediately of induction and hormone action. I would like to add to these the action of the vascular system on the population; there are also mechanical and nutritional, even nervous, influences to be considered. In the last analysis, all these influences may well have a final common path through the cell nucleus; at present we are at the stage of cataloging the factors and their visible and chemical effects on the population.

Returning to the concept of bone-cell populations, I would like to emphasize the continuity of the populations. We do not very often see a bone as a whole or think about its cell population in toto. In histologic preparations of small bones from young animals, one does get the idea of a continuum of cells within subpopulations in the periosteum, the bone marrow, the cortex, the metaphyses, and the epiphyses. When we discuss the characteristics of these subpopulations we would do well to remember that they are part of a continuum which starts from the periosteum, goes through the bone cortex, and on up and down the marrow into the metaphyses.

Let us consider bone from the point of view of population. A young bone has a fairly open cortex—that is, the periosteum—with part of its bone-cell population, the periosteum, in place. This is continuous with the bone-cell population, working through the interstices of the cortex. Then there is the population of the bone marrow through and including the special population of the endosteum at the surface of the bone marrow. Then we have the population going up into the resorption spaces, and then we have an independent population of the bone cells in the epiphysis. While this is an independent population, all the rest are really forming a continuum; there is no very great difference, in many cases, between the periosteum population in the cortex, the population within the marrow, and the population in the metaphysis. I think we were trying to differentiate between metaphyseal bone, cortical bone, and cancellous bone as if each were an entirely different species of bone. I think, in reality, if we trace their development we could see how the appearance of the bone gradually hardens and crystallizes out from a fairly basic unified pattern.

In any section of bony tissue taken from a young animal, one is liable to be confronted with a bewildering variety of cell types. There are plump osteoblasts and multinucleated osteoclasts on bone surfaces, and osteocytes of different kinds imprisoned within the bone matrix. There are "fibroblasts" in the outer layers of the periosteum and in the connective tissue around the larger blood vessels of the bone marrow. There are endothelial cells of blood vessels, leukocytes and erythrocytes in all stages of maturation, and fat cells. In the chicken bones I have been studying there are masses of nucleated erythrocytes in the blood vessels, which adds to the confusion. In addition to all this, there are certain nondescript, unspecialized-looking cells to be seen almost everywhere; these cells do not fall into any of these categories. They are present in the depths of the periosteum, around the fine vascular channels of cortical bone, in the spaces of cancellous bone, lining the medullary surface of the cortex, and scattered throughout the bone marrow. They have been given many names, and they have long been regarded as a reserve of uncommitted cells from which new osteoblasts

are recruited during growth and repair. Their status has been made much clearer since the introduction of tritiated thymidine as a nuclear label. It is now realized that these reserve cells can multiply and differentiate into a variety of specialized cell types including osteoblasts, osteoclasts, and chondroblasts.

In mature bone the case is quite different. Plump osteoblasts and osteoclasts are found only occasionally, most bone surfaces are covered with exceedingly flat cells, and the reserve cells mentioned are very difficult to identify. Yet if mature bone is stimulated by injury, for example, its cell population rapidly takes on the appearance it had in its youth. The flat cells rapidly fatten into osteoblasts, and near them reserve cells become conspicuous. Osteoclasts also soon make their appearance. It would seem that the cell population can go into hibernation, and then be awakened.

If we grant that bone contains a ubiquitous population of multipotential reserve cells, then the consequences of stimulating them to divide and to differentiate will be far reaching. Indeed, the obvious method of regulating bone activity would be exerting control over the multiplication and differentiation of these reserve cells. I should expect that much of our discussion will center around these cells and the factors which regulate their activity. But first perhaps we should agree on a generic name for them.

URIST: Would you call them mesenchymal cells?

PRITCHARD: I do not think that the name is as important as knowing that they are there. I think it is more important to delineate them by definite properties—what they can do, what they cannot do, how they stain, and so forth.

URIST: If the animal had one injection of tritiated thymidine, where would the label be located?

PRITCHARD: I suspect it would go into some members of the reserve-cell population in the first few hours.

URIST: The ^{3}H-thymidine would not go into the endothelial cells in any great amount.

PRITCHARD: No; because they are not about to divide.

URIST: The next layer, the nondividing cells, would not utilize a large amount of ^{3}H-thymidine. Only the population of cells, the progenitors, preparing to divide rapidly and in large numbers would be expected to be labeled with ^{3}H-thymidine.

PRITCHARD: But you would not know it was about to divide unless it took up thymidine and told you so. Otherwise, you probably would not notice it.

BÉLANGER: I think we know what our chances are for seeing the thymidine go to one area or another by the amount of mitotic figures which we can observe in a normal preparation.

PRITCHARD: Where does it go to? You mean in relation to the bone as a whole?

BÉLANGER: Yes. I am trying to get an answer for Dr. Urist. The use of thymidine is a very sophisticated approach to this kind of thing. Before thymidine was available it was possible to predict where it would go proportionately by the amount of mitotic figures that we could observe, particularly after colchicine, a very simple technique that anyone can do.

PRITCHARD: This comes under the question of the criteria we are going to use to differentiate different types or modulations of cells in a population. Could we keep to static considerations for a while, and deal with dynamic aspects later?

When you administer thymidine and label a cell, that presumably is a static study. You do not actually see the cell dividing; you merely have evidence that it is about to divide or that it has divided.

On this basis, Dr. Bélanger, what types of cell would you recognize in a bone population?

BÉLANGER: Do you mean in relation to thymidine?

PRITCHARD: Yes; in relation to thymidine. You can divide the population in many ways: in relation to phosphatase, in relation to glycogen, in relation to size and shape of cells. However, in relation to thymidine, what cells would you recognize?

BÉLANGER: I think we should ask Dr. Young, who has firsthand information on this sort of thing. I think we agree on some aspects about that. Some people do not agree, I believe, on the actual names which are given to these cells, but we all agree that they are what you call reserve material, what Cronkite et al. (ref. 108) call primitive cells, what Young calls osteoprogenitors (ref. 109), and what embryologists like to call mesenchymal cells; they are all the same. These are the cells in which you would expect most of the thymidine to be located—in all those areas, including the endothelial wall where there is a very rapid renewal of the cell population from other endothelial cells.

PRITCHARD: Do they remain endothelial?

BÉLANGER: I do not know, but certainly the endothelium renews itself in great part, except wherever new vessels appear from the mesenchyme from other cells in the wall.

PRITCHARD: Dr. Young, would you talk about the distribution of tritiated thymidine in a bone-cell population, without perhaps at this stage going into family histories and so on, but sticking to what you actually observe when you use thymidine?

YOUNG: My experience has been that one can distinguish at least four functional states of the bone cell. These states would include the three classic cell types: osteoblasts, osteoclasts, and osteocytes.

In addition, there is a pale-staining, rather spindle-shaped, cell which so far we have been hesitant to name. I call this cell the osteoprogenitor. It utilizes tritiated thymidine in synthesizing DNA prior to cell division and serves as a source of new bone cells.

The classic cell types are, generally speaking, incapable of reproducing themselves. However, the bone cell is capable of assuming a specialization in which it can divide, and it is in this state that it takes up thymidine.

While we are on the static aspect, I would like to show some figures which indicate that although (I will develop this later if time permits) the three classic cell types are all ultimately derived from cells produced through mitosis of the osteoprogenitor, they are nevertheless functionally different.

PRITCHARD: Is that static? I would have thought that was a dynamic problem.

YOUNG: I have proof for this statement, but have only mentioned it now as a background for the figures I will show.

If we examine, by autoradiography, the specialized cells in bone ½ hour after injecting tritiated glycine, we immediately see that these cells are functionally different (ref. 110). Figure 60(*a*) is an osteoclast which has utilized, relatively speaking, practically none of the injected glycine; whereas the osteoblasts in the very immediate vicinity, having access to the same tissue fluid, have concentrated the glycine in their cytoplasm in the act of protein synthesis. Glycine shows the greatest contrast between these two cell types because it is the most common amino acid in collagen, which is, of course, what these osteoblasts are largely synthesizing.

PECK: What is the evidence that glycine is in protein? There are many functions for amino acids in a cell besides incorporation in protein.

YOUNG: It can be shown biochemically that if one supplied these cells with tritiated glycine or ^{14}C-glycine, the radioactivity can shortly thereafter be recovered in collagen; but autoradiographers using this sort of technique have to accept the fact that practically all low molecular weight compounds are washed out in preparation of the sections. The result is that we are looking, in a sense, at a nucleoprotein carbohydrate residue of what used to be there.

Within 4 hours, most of this labeled protein is released from the cells (fig. 60(*b*)). The osteoblasts are now essentially devoid of radioactivity, and they have laid it down on the surface of the matrix.

Osteocytes, which are, after all, just trapped osteoblasts, also use tritiated glycine (fig. 60(*c*)); at least the new ones do, such as we saw yesterday in the elegant micrographs presented by Dr. Robinson.

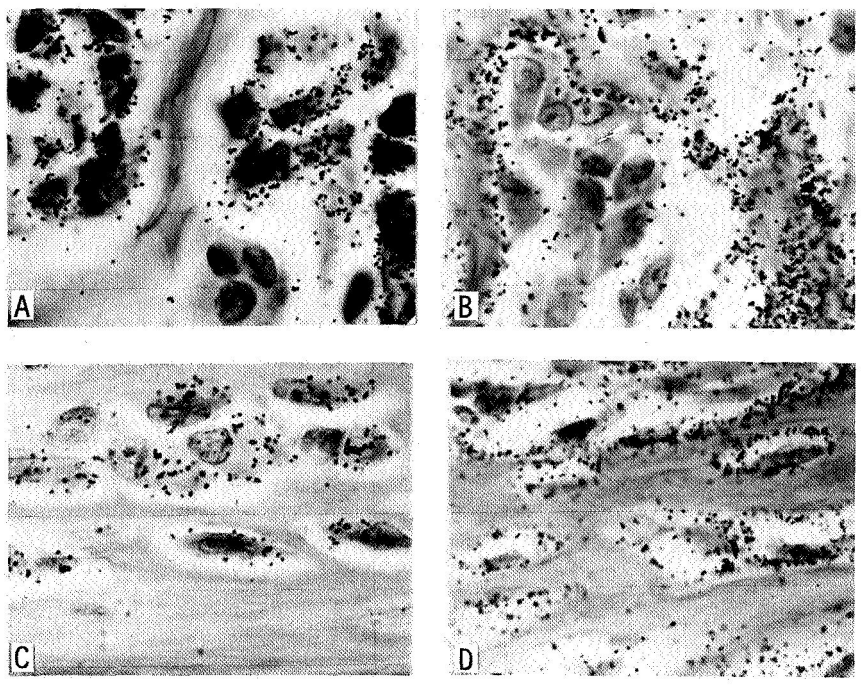

FIGURE 60. Photomicrographs of sections from tibial metaphysis of 1-week-old rats, sacrificed 30 minutes after injection of ^{3}H-glycine. Autoradiograph, PAS-hematoxylin stain. 900 ×.

(*a*) Osteoblasts have concentrated large amounts of the labeled amino acid. Note that the osteoclast (below, center) has incorporated very little of the radioactive protein precursor.

(*b*) The osteoblasts have deposited radioactive protein on the surfaces of the adjacent bone trabeculae.

(*c*) Newly formed osteocytes (above) are actively synthesizing protein. Older osteocytes (below) are relatively inactive in this regard.

(*d*) Newly formed osteocytes have deposited the radioactive protein on the surface of their lacunar walls.

These cells are also synthesizing bone matrix. The older osteocytes, which are smaller and in smaller lacunae, use little or no glycine.

Within 4 hours, the newly formed osteocytes lay down the radioactive matrix on the surface of their lacunae, which they are remodeling into smaller, more almond-shaped cavities (fig. 60(*d*)).

BÉLANGER: Is this a rat, Dr. Young, or a mouse?

YOUNG: This is rat bone. I have been talking here about glycine. We have also looked at many additional amino acids (ref. 111). From these figures, one might draw the conclusion that the osteoclasts and osteoblasts were doing the same thing, but at different rates. However, if we try a large series of precursors, we will find that the labeling

relationships between osteoclasts and osteoblasts differ. They are handling each precursor according to their own preference.

With some precursors, such as proline, there is still a high ratio of labeling in osteoblasts as compared with osteoclasts. In others, such as alanine (fig. 61(*a*)), the distinction is perhaps not quite so great.

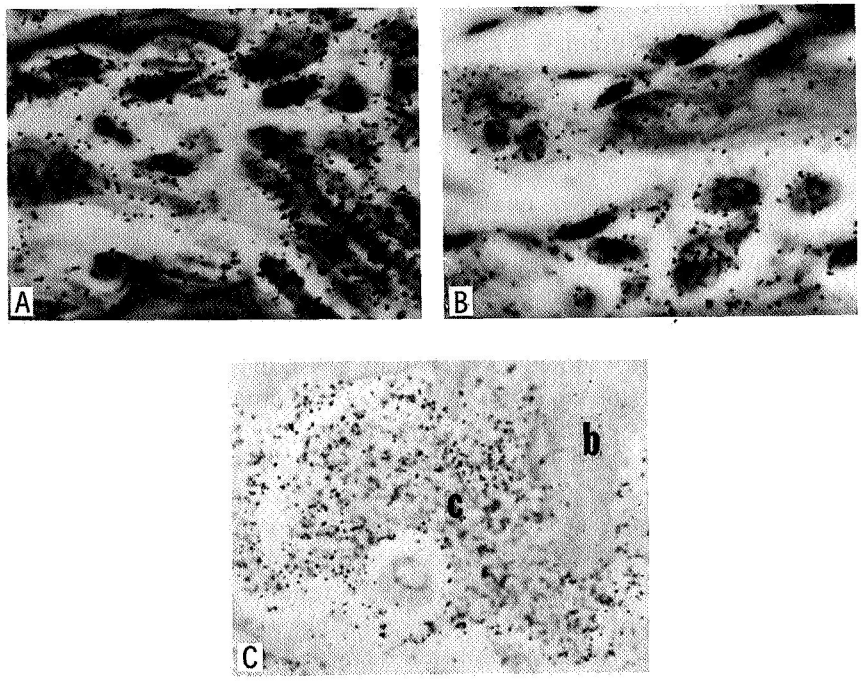

FIGURE 61. Photomicrographs of sections of tibial metaphysis of rats after injection of tritiated compounds. Autoradiograph, PAS-hematoxylin stain. 900 ×.

(*a*) One-week-old rat, sacrificed 30 minutes after injection of ^{3}H-alanine. Difference in labeling between osteoclast (left) and the osteoblasts is not as marked as with glycine and proline.

(*b*) One-week-old rat, sacrificed 30 minutes after injection of ^{3}H-histidine. Difference in labeling between osteoclast (left) and osteoblasts is even less.

(*c*) Eight-week-old rat, sacrificed 1 hour after injection of ^{3}H-taurine. Large, heavily labeled osteoclast (*c*) on surface of unlabeled bone matrix (*b*) appears to be engulfing an unlabeled cell (below, center). Glutaraldehyde fixation.

With histidine (fig. 61(*b*)), the labeling in osteoblasts and osteoclasts is fairly comparable, although on a per nucleus basis it is still higher in osteoblasts.

In some cases, such as this unusual precursor (fig. 61(*c*)), we find that the osteoclasts are very strongly radioactive, and the osteoblasts are relatively less heavily labeled.

This label is taurine; it is not an amino acid. It is believed to be a waste product, a breakdown product of the sulfur-containing amino acids. It is actually the first precursor that I have found which appears to be preferentially concentrated in osteoclasts.

I think that although we are able to distinguish these cells morphologically, these differences in appearance reflect underlying differences in the metabolic organization of the different functional states of the bone cell.

NICHOLS: Dr. Young, is it not true that the osteoblasts may be active in this sense in some areas of the bone, and in other ways not so active? Can you really use amino acid uptake as a reliable criterion for identification?

YOUNG: I have never observed a cell which was a full-blown osteoblast with well-developed endoplasmic reticulum, Golgi complex, and so on, that did not avidly concentrate protein precursors. One expects to find osteoblasts that are beginning to respecialize, perhaps as osteoprogenitors, or osteoprogenitors that are beginning to specialize as osteoblasts. These will not show the full response, the highest rate of bone formation. However, generally speaking, any cell which is histologically recognized as an osteoblast will, upon autoradiographic analysis, prove to be synthesizing bone matrix.

HOWELL: How old are the animals that you studied?

YOUNG: These are young animals. In old animals in a region of quiescence in bone, the osteoblasts are not fully developed; they tend to be flattened. They will show some uptake because they are indeed alive, their enzymes are turning over and so on; but here again the morphologic picture, for those who are not using autoradiography, can serve as a fairly good guide to the activity of the cell.

BÉLANGER: Is there a parallel between the cells that pick up thymidine and those that pick up the amino acids?

YOUNG: All cells that are alive are going to use some of the amino acids. Those that have reorganized their metabolic machinery in order to synthesize collagen are nondividing. Dr. Holtzer would agree with me, I think, that the cells which are highly specialized for some specific function are generally not dividing cells.

NICHOLS: A couple of years ago Dr. Owen made some autoradiographs of bits of pig bone which I had incubated with tritiated proline. We observed quite active uptake in the osteoblasts in some areas and in other areas practically none. We also observed labeling of osteocytes similar to that with glycine. Dr. Owen, is my memory correct?

OWEN: Yes. However, I would like to make one point. In very young animals (about 1 week old), such as Dr. Young and I have used, all the osteoblasts are actively synthesizing collagen. In older

animals only selected regions of osteoblasts synthesize collagen; other regions are quiescent. Dr. Nichols' material consisted of bone chips from older animals.

NICHOLS: All right, but what I am trying to get at is that an osteoblast can be considered an osteoblast, at least by a biochemist, and still not be making much collagen at the time. I think that this is an important point because I have the notion that differentiation can occur without the cell necessarily having to perform the function for which it is now differentiated. In other words, a runner does not necessarily have to be running all the time to be recognized as a runner. He can occasionally stop.

RAISZ: Were those precursors given *in vivo* or *in vitro*?

NICHOLS: *In vitro.*

RAISZ: I would object to the interpretation on the ground that the function of some of the cells may have altered since they were removed from the animal.

TALMAGE: I am sure there are differences in the rate in which various osteoblasts take up protein and synthesize collagen. For example, it has been demonstrated in the rat that osteoblasts in the metaphyseal region of the femur turn over radioproline at a much faster rate than do osteoblasts in the shaft of the same bone.

NICHOLS: We can demonstrate very clearly that proline went through the osteoblasts in the metaphysis at a much faster rate than it did through the osteoblasts in the cortex of a rat bone. After one-half hour a fair amount of metaphysis had gone into collagen whereas in the diaphysis, 96 percent was still left in the cell. We followed through to 4 hours and were able to draw a curve on the rate in which osteoblasts in different parts of the bone were synthesizing proteins.

PRITCHARD: As a matter of fact, we are trying to get to the criteria for distinguishing species of cell in the population. These different cell types are going to vary in their activity from time to time. Using the criterion of labeled amino acid uptake, there appear to be differences between the kinds of cell which morphologically we term osteoblasts, osteoclasts, and marrow cells.

Metabolic criteria are important, but we must not forget that there are other ways of distinguishing the elements of this population. As I have indicated, shape, size, and gross internal structure were all that the classic morphologist had to go on for a number of years. When he saw a cell with long, thin processes, with a large nucleus at one end, an enormous negative Golgi area in the middle, a basophilic cytoplasm full of mitochondria, and when that cell was sitting on a bone surface, he called it an active osteoblast.

When he saw a much larger cell with many nuclei, a large number of mitochondria, and residing in an excavation on the bone surface,

he called it an osteoclast. He forgot to talk about or only mentioned in passing, as a rule, the other cells present, because they did not have these very distinctive morphologic features.

Then there are staining reactions. The cytoplasm may be either eosinophilic or basophilic, or a mixture of the two.

Another criterion, of course, is enzyme activity. There seems to be no limit to histochemical criteria. One cell type may have very high alkaline-phosphatase activity, whereas another cell type may have a very high acid-phosphatase activity. Succinic dehydrogenase activity may be high in one type of cell, and in another type there may be low isocitric dehydrogenase activity and so on.

We have not yet mentioned tritiated thymidine, which is mainly used to determine family relationships within a dividing, differentiating population. But thymidine is also a static criterion in that we can distinguish certain cells that take up thymidine immediately from those that do not. I am sure there are many other physical and chemical criteria waiting to be tried. We certainly have enough criteria. Are we in a position to classify the cell types and agree on names for them? What shall we call the cell that lies on a bone surface looking like an osteoblast that has been flattened? It still has a nucleus at one end, it still has the big negative Golgi image, and it still is basophilic; but it is as flat as a pancake. This cell type is rarely mentioned.

Nevertheless it is ubiquitous and, moreover, I have seen many transitions between this type of cell and the plump classic osteoblast. I would like to suggest that the flat cell is a resting osteoblast, and the plump cell is an active osteoblast. It seems that when an osteoblast has stopped being very active, it may not necessarily go back to a progenitor stage but may just go back to a resting stage, while retaining its potential as a working cell.

BÉLANGER: It is a very important point, Dr. Pritchard, if I may say so, that not all osteoblasts become osteocytes. Some become resting osteoblasts. I do not know now whether I agree with Dr. Young that they may go back to being mesenchymal cells or young cells, but some active osteoblasts, a fairly large number—depending on where we are looking in the bone and depending on the actual growth potential of the site—will go into the resting state; we can follow the thymidine through time into these or into osteocytes. In other words, the number of osteocytes does not account for the number of osteoblasts which showed the thymidine previous to that stage.

SAXÉN: Could you define the resting stage?

BÉLANGER: This is the particular stage that Dr. Pritchard discussed.

PRITCHARD: Most bone surfaces in the adult are covered with a pavement of flat cells. These cells have certain osteoblastic features. Their long processes go into the bone, the nucleus is at one end, and

there is a large central Golgi zone; but they do not have the amount of cytoplasm or the number of organelles. They are literally at rest, but there is no evidence that they are engaged in bone formation or resorption. These are the cells that we need to name. I call them resting osteoblasts.

NICHOLS: Does anybody have an electron micrograph of one?

PRITCHARD: Dr. Robinson showed them.

OWEN: Our studies of the kinetics of cell differentiation on the periosteal surfaces of young rabbit femur may be of interest at this point. We have measured the rate at which preosteoblasts—perhaps I should not use that term——

FREMONT-SMITH: Why not?

OWEN: I mean the rate at which the precursor cells of the osteoblasts differentiate to become osteoblasts, and then the rate at which the osteoblasts go on to become either osteocytes or osteoblasts living in haversian canals. We found no evidence of any cell death in this system. All osteoblasts originally on the surface eventually became either osteoblasts in haversian canals or osteocytes within the matrix, about 60 percent and 40 percent, respectively, in the two categories (ref. 112).

FREMONT-SMITH: No resting cells?

OWEN: The osteoblasts in haversian canals, especially those deep within bone, are resting osteoblasts.

PRITCHARD: Dr. Owen, would you tell us what your criteria were? I have given my criteria for the population of resting osteoblasts. What would your criteria be?

OWEN: In our particular study it was not necessary to define resting osteoblasts. However, I am more or less in agreement with your definition. In our material, haversian canals that are deep within the bone have osteoblasts on their surface which I would describe as resting. The cells are flattened against the surfaces of the lumen and do not show detectable labeling with tritiated glycine.

PRITCHARD: In other words, location, rather than any specific morphologic feature.

OWEN: Yes; in this particular study the main criterion was location.

MCLEAN: What about the alkaline phosphatase?

OWEN: I would like, at some time, to put in a plea for the term "preosteoblast," because I think there may be several stages included in the osteoprogenitor stage of the cell. Balogh and Hajek (ref. 113) found different histochemical staining reactions in osteoprogenitor cells in different situations. In their studies, what they describe as the osteoprogenitor cells of the periosteum show a moderate staining activity for isocitric dehydrogenase and glucose-6-phosphate dehydrogenase, whereas these enzymes were not demonstrable in the

osteoprogenitor cells of fracture callus. They also describe, in fracture callus studies, mononucleated cells showing succinic dehydrogenase reactions; the enzyme specific for osteoclasts in bone. They suggest that these mononuclear cells may be precursors of the multinucleated osteoclasts—might I suggest the term "preosteoclast." Walker (ref. 114) also described mononuclear or binuclear cells with strong succinic dehydrogenase activity in bones treated with parathyroid hormone. If the proliferating precursor stage of bone cells—the osteoprogenitor stage (ref. 109)—does consist of several stages, this would fit well with what has been found in other tissues; for example, the well-known multiple stages of proliferating precursor in the blood series. More recently Combs et al. (ref. 115) reported several stages which were histochemically and autoradiographically distinguishable of proliferative precursors in mast cells.

URIST: Does Dr. Owen propose that a gradation of changes in cell metabolic reactions could occur in the course of mitotic division?

PRITCHARD: She did not really say that.

URIST: I will restate the question. There is a series of mitotic divisions, and there is an arrangement of cells in layers. The layers consist of perivascular connective tissue cells, mesenchymal cells, and connective tissue cells closest to the bone, which are preosteoblasts. In my mind this raises the question of whether another criterion, the one Dr. Nichols mentioned, that of ultrastructure, can rescue us from the dilemma of morphology under the light microscope.

Can Dr. Robinson and his associates, who are familiar with these cells in the electron microscope, distinguish between a perivascular connective tissue cell in a muscle and a perivascular connective tissue cell in a bone? I think that is what I am searching for in order to identify a cell by its potential for function.

PRITCHARD: We are trying to arrive at some objective, incontrovertible criteria for saying, "This is an X cell; this is a Z cell; this is a dead cell."

URIST: Will the criterion of ultrastructure contribute something in addition?

PRITCHARD: I think it will help, but we need evidence from many different sources; we will have to say where the different criteria overlap and congeal into definitive species, like separating out the animals of an animal population. One criterion does not, in general, enable you to distinguish one species from another. You have to integrate a lot of criteria before you can say, "This is one species, quite distinct from that species."

URIST: Dr. Robinson, can you distinguish between a preosteoblast and a premuscle cell? The two can be found in adjacent areas in every section of bone tissue.

ROBINSON: No.

URIST: There is no ultrastructural characteristic?

ROBINSON: I am not saying there is no ultrastructural characteristic. It is just that I have not studied the difference between a preosteoblast and a premuscle cell.

URIST: Let us say a preosteoblast and a prehematocytoblast. They are all right there.

ROBINSON: Well, I think that one of the things that has fascinated us in the study of the haversian canal is that there is really a three-cell layer, even in the young canal; there is the endothelial cell, the cell that lies under the endothelial cell, and the cell that lies next to the bone. The cell that lies between the endothelial cell and the bone cell has some characteristics of both, if you want to speak about morphology of fine structure of the cytoplasm.

This is described by Cooper et al. (ref. 25), but we are not sure what these slight resemblances mean because, after all, the morphology may have something to do with the function of the cell at the moment.

What I am interested in now is where these osteoblasts can possibly come from in many sites in bone; for instance, up in the area where new bone is forming on trabeculae of calcified cartilage. Trueta (ref. 116) has pointed out that the only cell around seems to be the endothelial cell, and he, I think, stated quite definitely that the endothelial cell became an osteoblast.

PRITCHARD: Many people would not agree that there are no other cells around, that the endothelial cell is the only candidate. They would point to the mesenchymal population around the blood vessels.

ROBINSON: Well, we felt quite differently than Trueta did. We felt that the endothelial cell might give rise to the precursor of the osteoblast, but we were in doubt as to the origin of the endothelial cell itself. Could it be one of the functional forms of a monocyte?

I would like to ask Dr. Owen about statements in her paper in which it was mentioned that thymidine was picked up by the endothelial cells, by the progenitor of the osteoblast, also once in a while by the osteoblast, and even by the surface osteocytes, cells which I do not think would be expected to divide. I think Dr. Owen referred to Pelc in this regard.

OWEN: I think you are referring to the paper (ref. 112) where we were studying the growing surface of young rabbit femur. The osteoblasts are on the bone surface; the precursors of the osteoblasts, termed the "preosteoblasts," are behind the osteoblasts. These latter cells were the main region of thymidine uptake at short times after injection. They may well have included endothelial cells; we did not try to distinguish these. We never found early thymidine labeling of osteocytes. Labeled osteocytes were found, but at later times, due to the fact that

the cells had taken up the thymidine at an earlier stage, probably as preosteoblasts.

BÉLANGER: Certainly, migration.

OWEN: Migration, yes. Perhaps you are referring to a second paper; there were two papers. Are you referring to the first or second?

ROBINSON: I am referring to the paper by Owen and MacPherson (ref. 117).

OWEN: Yes; that was the second paper. As you know, incorporation of thymidine into DNA occurs during the process of doubling of the cell's DNA in preparation for cell division. All cells that have taken up thymidine should have divided by about 17 hours after injection. We found that a certain proportion of the cells that took up thymidine did not appear to have divided by one, two, or more days after injection. Our evidence came from grain-count studies where we showed that a proportion of the cells at later times had the original grain count of cells 1 hour after thymidine injection. As yet we have not made any further progress on this matter, and I do not know what its significance is.

PRITCHARD: Does this include the osteoblasts?

OWEN: A small number of osteoblasts do take up thymidine; other people have also reported this. However, this could possibly be explained in terms of our criterion for distinguishing preosteoblasts from osteoblasts. We distinguish in terms of location only so that a labeled osteoblast could in fact be a preosteoblast in the wrong location.

BÉLANGER: Dr. Owen, did you not use the double-labeling method at one time to show a label in the nucleus and a label in the cytoplasm—let us say, protein synthesis in cytoplasm and nuclear labeling from thymidine?

OWEN: I think you are referring to our experiments using tritiated glycine.

BÉLANGER: I think what you did was to show incorporation in the matrix from cell.

OWEN: Yes. Glycine is taken up into collagen; i.e., glycine is first incorporated into the osteoblast and then laid down in matrix collagen. In addition, as Dr. Young showed, there was also some uptake of glycine into young osteocytes. These are the osteocytes near a growing bone surface, and in more recent work I have found that they also take up a little RNA.

YOUNG: RNA precursors?

OWEN: RNA precursors, at a low rate.

YOUNG: I think that the terminology has been perhaps a little obscure here. I feel that we should recognize in bone four major functional states of the bone cell. One of these is a dividing state, which I call the osteoprogenitor, and this osteoprogenitor, the offspring from this

cell, can specialize as either osteoblast or osteoclast. If given the opportunity, I will try to demonstrate that to you. That is why I feel it is misleading to call this dividing cell either a preosteoblast or preosteoclast because it implies that you know, before it has specialized, how it is going to specialize.

Now, if the osteoprogenitor can specialize as an osteoblast, which it can, there must be a stage when it is accumulating the intracellular machinery that will ultimately characterize it as an osteoblast. During this specialization process, which is a matter of a few hours (ref. 109), it will have some of the characteristics of an osteoblast, but will not be a fully developed osteoblast. If we wish to use the term "preosteoblast" for this cell when it is in the process of specializing, I do not see any objection to that. However, I would like to point out that a problem arises if you are observing an osteoblast that is respecializing as an osteoprogenitor. You may call it a preosteoblast when it is actually postosteoblast.

PRITCHARD: This is a big question, reversibility. We must discuss reversibility at some time because it is crucial to the whole problem of cell physiology.

HOLTZER: Can I make a plea that this discussion, which really could have taken place 30 years ago, is still taking place, and——

PRITCHARD: We did not have any tritiated thymidine then.

HOLTZER: That is it. So let us forget the guidelines of 30 years ago and focus on Dr. Young's elegant model. There is a population of cells. Some of them can be identified under the microscope, others cannot. I think some of the questions raised, "Do they all work?" and "How can they be recognized in the process of transforming?" are very provocative, but how can they be approached experimentally?

PRITCHARD: I think we ought to communicate.

HOLTZER: We have an operational definition: At a given time a given cell is engaged in making A, B, and C kinds of molecules. At a later time can that same cell engage in making X, Y, and Z molecules? In brief, let us not worry about names; rather, let us be concerned about the experimental evidence which shows that before, during, or after a given mitosis, a cell synthesizes this or that kind of molecule. So many questions we ask cannot as yet be answered in terms of current methodologies.

PRITCHARD: All right. Let us leave the terminology at this stage by agreeing that underneath the fiber layer there is a progenitor layer. Then there is the next layer which the problem is really about; what are we going to call this layer, before we get down to the cells that everybody agrees on? What are we going to call these intermediate cells which do not multiply as fast as those of the other layer. The term

mostly used is "preosteoblast," but should there not be preosteoclasts and prechondroblasts? What should we call the intermediate cells?

OWEN: I have pointed out some differences that have been demonstrated within osteoprogenitor cells, but I do not want to emphasize them. For the moment I think Dr. Young's scheme is an excellent working basis: osteoprogenitor cells and the functional cells of bone including osteoblasts, osteoclasts, osteocytes, chondrocytes, and so forth. However, I am of the opinion that differentiation is probably not a one-step process. It is a gradual accumulation of characteristics until the fully differentiated cell is achieved. Eventually we will be able to distinguish the different states, which I am certain exist, among osteoprogenitor cells; but for the moment I propose that we stick to Dr. Young's scheme.

RAISZ: I would like one point clarified. Is cell division required to go from one of these cell types to another? This is the implication now, but from things said earlier, I did not think it was true.

YOUNG: Cell division is required to make new cells.

RAISZ: That is helpful. It is not necessary to divide in order to go from one to the other?

YOUNG: No.

OWEN: That is another question, another problem.

PRITCHARD: Please bear with me for a moment. This question of the relationship of these cells to each other and the criteria for differentiating them is not purely academic. Many advances in bone physiology depend upon getting the right answers, and we also have to establish a firm base for genetic studies. I should like to stress that Dr. Young is not the only man in the field so far as these relationships are concerned. Frost (ref. 118) has a scheme in which the cells go in one direction and all die at the other end, he has no reversibility in his system. Other people have modulation effects, side effects, one cell turning into another. Dr. Young's scheme is a reversible scheme. These differences are significant and important. I would like to know what the evidence is before we take up the more dynamic aspects.

BÉLANGER: I would like to object to the statement just made by Dr. Owen. I think the term "osteoprogenitor" was proposed by Dr. Young to apply to normal events only. If you have a fracture, for instance, you can see that these cells which are supposed to be under normal conditions, just progressively becoming bone cells, will now start making cartilage in the same site. Therefore I do not think that there is, at this moment, a very fixed destiny of the cell. This cell can divide. This cell is an ancestor of some kind; but depending on the local or general conditions of the organism, the cell could just as well turn into a cartilage cell, into a blood cell, or into all sorts of things. So I think that the old term "mesenchymal cell" or the term "stem

cell," such as is used by Cronkite et al. (ref. 108) in their excellent work, does not commit that cell to any specific destiny.

NICHOLS: I think the thing we really want to know now is whether one can identify these cells on a functional basis; and if so, how?

BÉLANGER: It is for Dr. Nichols' benefit, mainly, that I would like to answer that question and stress the point about the resting, or let us say "lazy" osteoblasts in relation to active ones. Figure 62 shows a "lazy" one seen at low power. We can see that it has already been growing processes next to the surface bone, but it has a fairly well-developed endoplasmic reticulum, and a few processes on the opposite side. But the main character of this cell, which distinguishes it from an active osteoblast, is the lack of development of the Golgi complex. This cell has practically no Golgi apparatus. If one can distinguish where, in time, this cell belongs in the lineage of migration of cells after

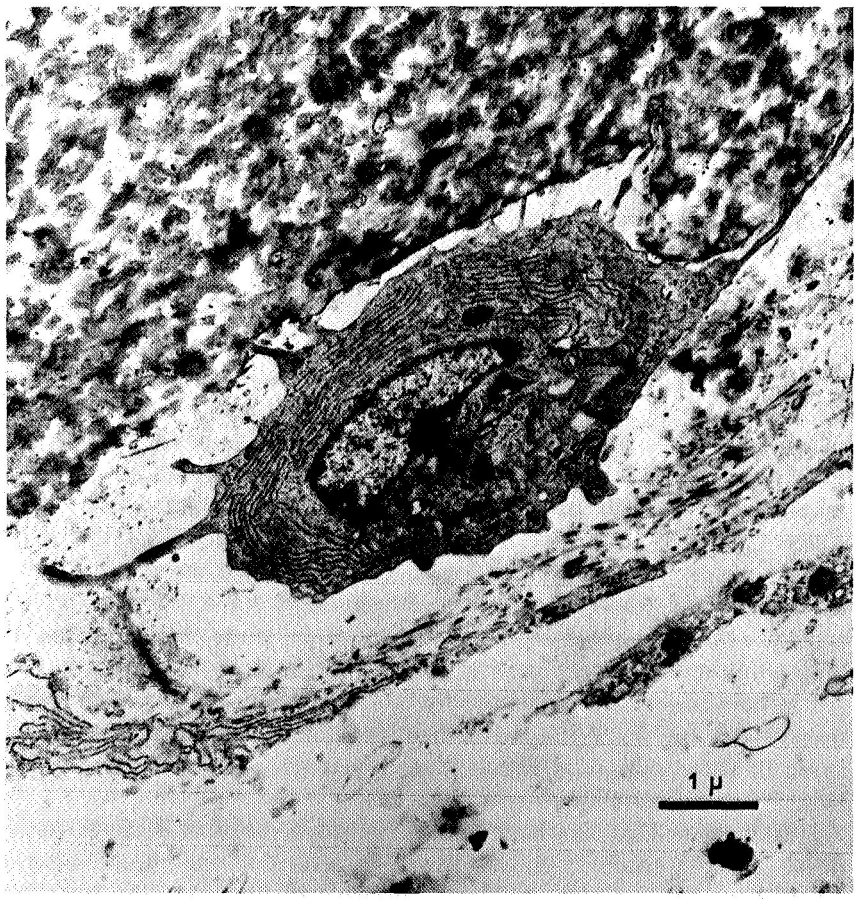

FIGURE 62. Osteoblast from tibial diaphysis of 11-day chick embryo.

it has picked up thymidine, this cell is not an active osteoblast. For some reason it is not manufacturing protein in large amounts, or other constituents of bone. It is a resting cell, but it is already a highly differentiated cell.

YOUNG: How do you know it is not synthesizing protein?

BÉLANGER: I do not say it is not. It does not show that it is active at the same rate as the large osteoblasts Dr. Pritchard has described in which one can see a very well developed Golgi apparatus and an endoplasmic reticulum with large cisternae. In this cell there are no cisternae and no Golgi complexes.

NICHOLS: Dr. Young, when you see osteoprogenitor cells, do they take up precursors, such as glycine or proline?

YOUNG: You show only a tiny slice of one cell. I am not convinced there might not be a Golgi complex in the next section.

BÉLANGER: This is a low-power thing, and I realize that you have not seen the whole cell; but Dr. Nichols asked for a distinction. The only distinction I could suggest would be that the Golgi net is small at the endoplasmic reticulum.

NICHOLS: I would like to comment on this discussion because I think it is very important for several reasons. I submit that morphologic criteria, such as presence or absence of rough endoplasmic reticulum, are not sound evidence in assessing the activity of the cell. The mere presence of equipment does not mean that it is being used.

The time required for a cell to get ready to make protein under a stimulus may well depend upon how much of the needed equipment it has; perhaps this is differentiation—at least in one sense. The point is that there can be various kinds of cells in various stages of activity depending on a whole series of stimuli. While it may be quite different to equate a given cell to a given job, this is going to be very important if we are going to understand (1) how the tissue is formed, (2) how it is taken away, and (3) what goes wrong with the system when we get sick.

PRITCHARD: Consider the flattened cells on the surfaces of adult bone. If you fracture the bone, these cells have become plump, typical osteoblasts and have started to make bone matrix within 12 hours. So they can get their machinery in top gear in 12 hours.

FREMONT-SMITH: Are there any tissue-culture studies which would throw light on this? Because then you can get moving pictures of some of these cells and watch them when they are doing it. Is any material on this available?

URIST: Yes; that has been done. When a piece of bone is put in tissue culture, there is an outgrowth of cells that are spindle-shaped connective tissue cells, that look like mesenchymal cells, but they exhibit a different capacity for development.

FREMONT-SMITH: Do you know where they come from?

URIST: We assume they come from osteoprogenitor cells. If you use a cataract knife to remove a bone explant from tissue culture and transplant the outgrowth back into a living rat, the cultured cells will differentiate into osteoblasts. Cells that look like undifferentiated connective tissue cells can be different insofar as they can exhibit osteogenic potency. If one looked at the spindle-shaped cell of the outgrowth under the electron microscope I do not know what one would see; I do not know anyone who has done this experiment to investigate the ultrastructure of the cells of the outgrowth.

FREMONT-SMITH: Some of the moving pictures of tissue-culture cells in the central nervous system, for instance, have changed our views so strikingly that I thought maybe this would answer some of the questions Dr. Nichols raised. I thought maybe we were using the wrong criteria for making decisions as to what a cell can do or has been doing.

NICHOLS: I am not acquainted with those observations so I cannot comment, but certainly one of the problems with tissue culture is dedifferentiation, which seems to be a particular plague of cultures of fibroblasts and connective tissue cells at large.

URIST: This is correct. Dedifferentiation does occur, after two and three generations of cell culture. Eventually the cells can no longer make bone. The more recently the cell has had contact with bone, the more likely it is to be able to make bone when it is grown out on tissue culture and returned to an intact animal.

FREMONT-SMITH: You mean only after the cells were transplanted back?

URIST: Yes; after the cell has been away from the animal and away from the bone for a long time, after the third and fourth generations have been cultured and recultured, the cells of the outgrowth lose their potency to differentiate into bone after transplantation.

PRITCHARD: Müller (ref. 119), one of the great pioneers of tissue culture, subcultured osteoblasts about 19 times, if I remember correctly, and yet they kept their potency. This does not agree with what you say. The cell may look different, but it keeps its potency.

NICHOLS: I submit that the cell does not even have to be near bone. I remember some experiments by Dr. Huggins in 1931, in which he transplanted bladder epithelium and got bone (ref. 120).

URIST: That is another interesting problem.

PRITCHARD: Surely, it depends on the environment. If you can put them back into the environment that they came from——

URIST: Eventually, after many cell divisions, the cell loses osteogenic potency. It may divide 19 times, but eventually it must lose the

capacity to produce bone. It may be a question of dilution of a genetic material or cytoplasmic substance.

HOLTZER: The main difficulty in this type of problem is cell transformation, or dedifferentiation, and cell selection. Whether cells in culture reversibly or irreversibly alter their metabolic behavior, or whether a cell type originally in the minority crowds out the cell type being studied, is a very confused issue. This problem can be avoided by beginning with a pure population of cartilage cells. Then by combining autoradiography and biochemical extraction procedures, one can at least pose the question of what some cells at a given moment are doing.

For example, without going into details, postmitotic cartilage cells taken out of their matrix can be induced to reenter the mitotic cycle and, in addition, to begin to synthesize a hyaluronic-acid-like molecule. Now, if after a few days they are allowed to aggregate, they cease making DNA and revert to synthesizing chondroitin sulfate. Alternatively, if the liberated chondrocytes are maintained as monodisperse cells in culture for several weeks and then allowed to aggregate, they do not go back to synthesizing chondroitin sulfate that can be detected as metachromatic matrix. In short, the chondrocyte's progeny has a fine memory for fabricating molecules required for mitotic activity, but its memory for synthesizing chondroitin sulfate, under certain conditions, is considerably more fuzzy.

PRITCHARD: It depends on the environment. If you can put them back into the environment that they came from——

HOLTZER: We did that and in the "normal" environment of the chorioallantoic membrane or the somite, they do not revert to making chondroitin sulfate. On the other hand, under conditions we have not used, they might resume their original or, for that matter, a quite novel metabolic activity. For, although the story is by no means complete, experiments on nuclear transplantations and on virus fused cells all point to a great deal of "reversible" behavior in mature cells.

URIST: Perhaps it is necessary to assume that the capacity to produce bone is lost only temporarily. Every connective tissue cell in the body may have the capacity to make bone, especially if it undergoes a series of mitotic divisions and is in a conducive environment.

PRITCHARD: That is an act of faith.

URIST: We will discuss the subject of potency again when we get to the induction systems.

Every connective tissue cell came from an original cell, so it is just a question of how far back we want to go to retrace the development from the unspecialized connective tissue cell to the osteoprogenitor cell.

PRITCHARD: Why do not liver cells make bone?

NICHOLS: Could we ask Dr. Holtzer to tell us more about his cartilage cell? Fundamentally, I agree with him; we ought to find out what cells are doing.

PRITCHARD: Dr. Holtzer says that in 12 hours they start making hyaluronic acid instead of chondroitin sulfate. Do they make collagen up to then? Could we see his figures?

HOLTZER: Many of the issues that have been discussed can only be analyzed critically with a homogeneous population of cells. Bone, at best, consists of a variety of cell types, and any measurements either of whole bone or on cells from bone, of necessity, involve measuring changes in the activity of more nonbone cells than true osteocytes or osteoblasts. By stripping away the adhering connective tissues from embryonic cartilages and treating such cartilages with trypsin to digest the matrix, a quite pure population of chondrocytes can be obtained; with care, well over 99 percent of the liberated cells are differentiated, working chondrocytes. Now, what these liberated postmitotic chondrocytes, which are making chondroitin sulfate and collagen, will do after removal from their matrix depends on how they are grown. In different microenvironments they synthesize different kinds of molecules. If, as already mentioned, liberated chondrocytes are spun down into a smaller cluster, they remain postmitotic and continue to synthesize chondroitin sulfate and collagen (ref. 121). Alternatively, if plated on top of a clot, they spread and are induced to reenter the mitotic cycle.

Figures 63, 64, and 65 show that in addition to making DNA and the other kinds of molecules, multiplying cells synthesize, dividing chondrocytes, and their progeny synthesize a polysaccharide rich in glucosamine, this polysaccharide is not sulfated and has the electrophoretic mobility of hyaluronic acid. Neither the *in vivo* chondrocytes from 10-day embryos nor the liberated chondrocytes spun down into pellets synthesize this hyaluronic-acid-like polysaccharide.

With time these cultures become more dense owing to cell multiplication. Correlated with this is a shift in the kinds of polysaccharides these cells produce. A polysaccharide appears with the mobility of hyaluronic acid but with a high galactosamine-to-glucosamine ratio (ref. 122). In addition, large amounts of chondroitin sulfate are made in the new dense cultures.

If the progeny of chondrocytes are grown for a considerable time *in vitro* and then challenged to display their capacity for synthesizing chondroitin sulfate, the results again depend on the nature of their *in vitro* microenvironment. For example, as shown in figure 66, if grown in reasonably high densities for four generations and then spun down and grown as cells in a pellet, the progeny of chondrocytes make

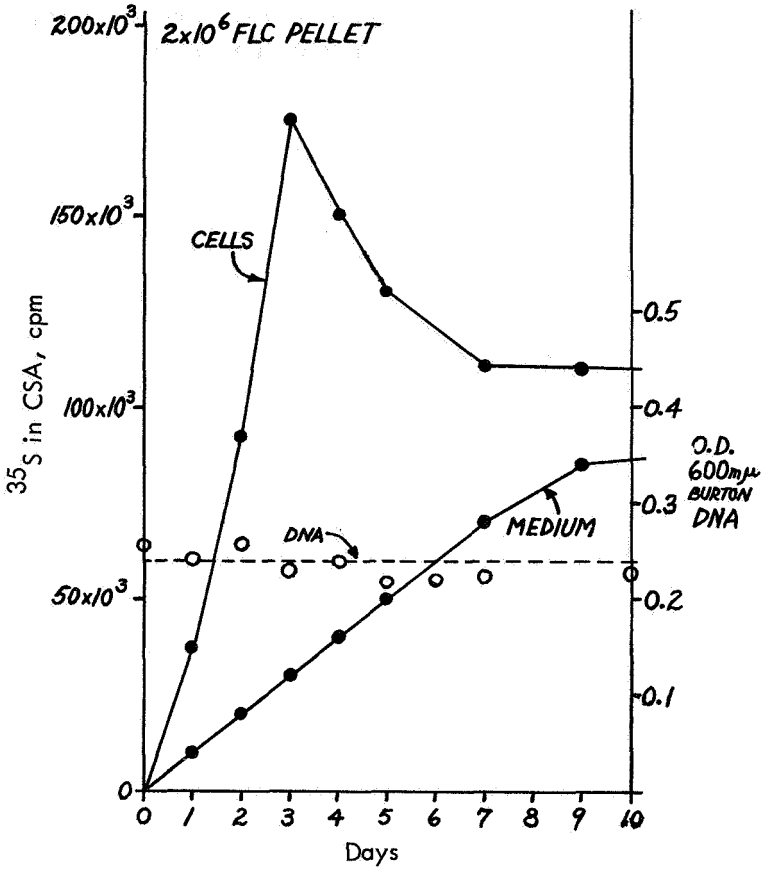

FIGURE 63. Freshly liberated chondrocytes (FLC) were spun down into a pellet and organ cultured in ^{35}S. The tissues were harvested on different days and the counts in chondroitin sulfate (CSA) determined. Under these conditions there is no net synthesis of DNA and the chondrocytes continue to make chondroitin sulfate. In other experiments it has been shown that they continue to make collagen as well.

much less chondroitin sulfate per unit of DNA than freshly liberated chondrocytes grown under identical conditions.

To check whether this diminution is a result of all cells making less chondroitin sulfate per cell or whether some cells are not producing while a minority are working hard, we performed cloning experiments (ref. 123). Without going into details of culture procedures, we grew in the same dish in the presence of ^{14}C-glucose, colonies of matrix producers and colonies of dedifferentiated, or transformed, chondrocytes. The matrix producers synthesized chondroitin sulfate, and the transformed cells produced a different spectrum of polysaccharides.

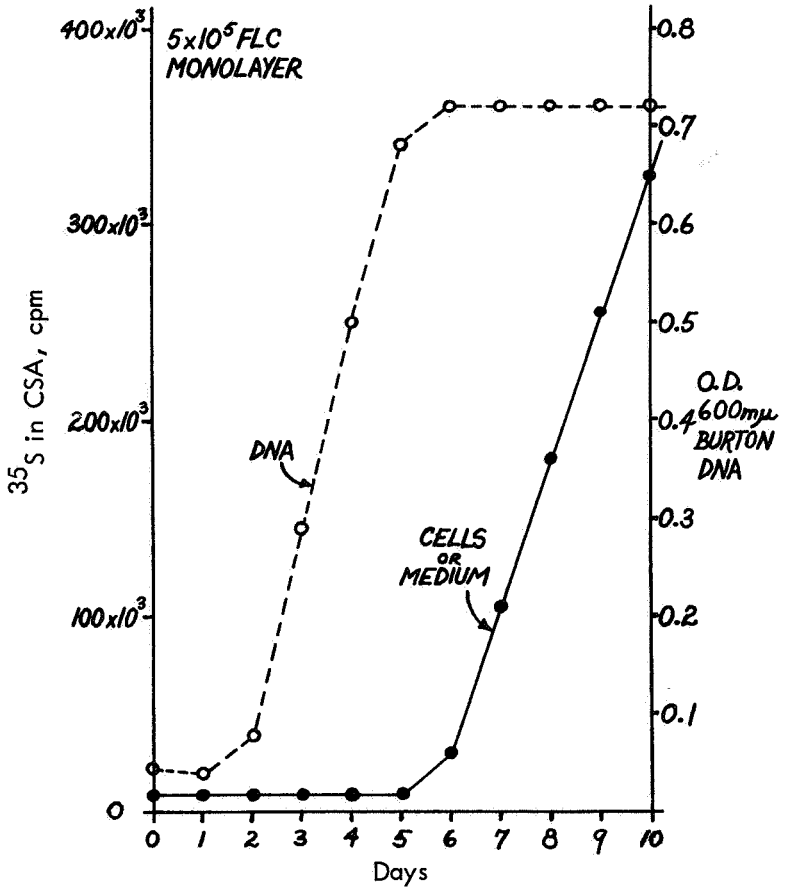

FIGURE 64. FLC cells were plated in milk bottles on top of a clot as monodispersed cells. There is an abrupt rise in DNA synthesis during the first 4 days of culture, which levels off after a certain density is reached. About this time the cells resume the synthesis of chondroitin sulfate.

From this we suspect that the chondroitin sulfate made in the fourth-generation cultures (figs. 66 and 67) might be made by a minority of the cells in the cultures; the majority are dedifferentiated, or transformed, chondrocytes. Thus far we have not been successful in shunting the dedifferentiated, or transformed, chondrocytes back into chondroitin sulfate producers.

These clonal experiments (ref. 121) led to another finding. When chondrocytes are cloned on plastic they may organize into an epithelial sheet which morphologically and functionally serves as an *in vitro* perichondrium. Cloned cells on plastic divide and, because daughter cells do not migrate, establish compact epithelial colonies. By mitosis, new cells are added to these tight little islands both in the plane of the

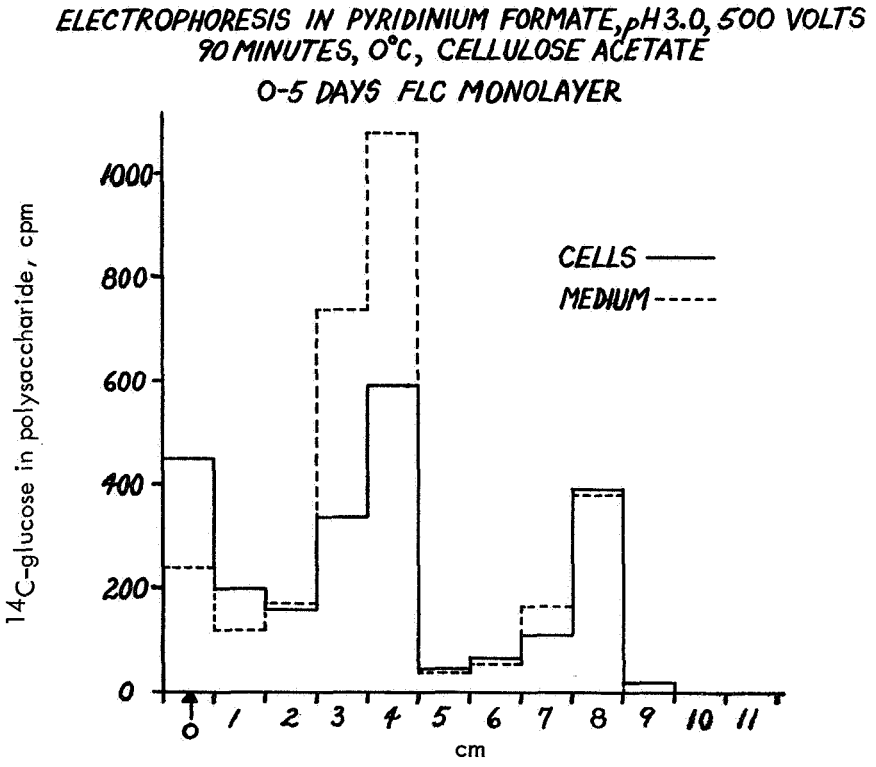

FIGURE 65. FLC cells were grown as monodispersed cells in the presence of ^{14}C-glucose. After Pronase digestion the polysaccharides were separated in pyridinium formate on cellulose acetate. Observe the prominent peak where hyaluronic acid would run. On hydrolysis this peak yields a fraction with a high glucosamine-to-galactosamine ratio.

substrate and also displaced upward from the substrate. The displaced cells become rounded and synthesize chondroitin sulfate. The same cells on a fibrin clot spread, do not form epithelial colonies, and do not synthesize chondroitin sulfate in appreciable amounts. If, after growing on a clot for five generations, these cells are cloned on plastic, they fail to form epithelial colonies and fail to synthesize detectable amounts of chondroitin sulfate.

The central question to which these kinds of experiments are directed is, What kinds or species of molecules can a cell make concurrently? For example, I do not believe that a cell can make myosin and albumin simultaneously, that the cytoplasmic-nuclear conditions required for the one preclude the fabrication of the other. On the other hand, a cell synthesizing myosin does make the cytochromes, dehydrogenases, myoglobin, ribosomal proteins, glycogen, and so forth. But it is by no means clear that all kinds of molecules found in a given cell can be made

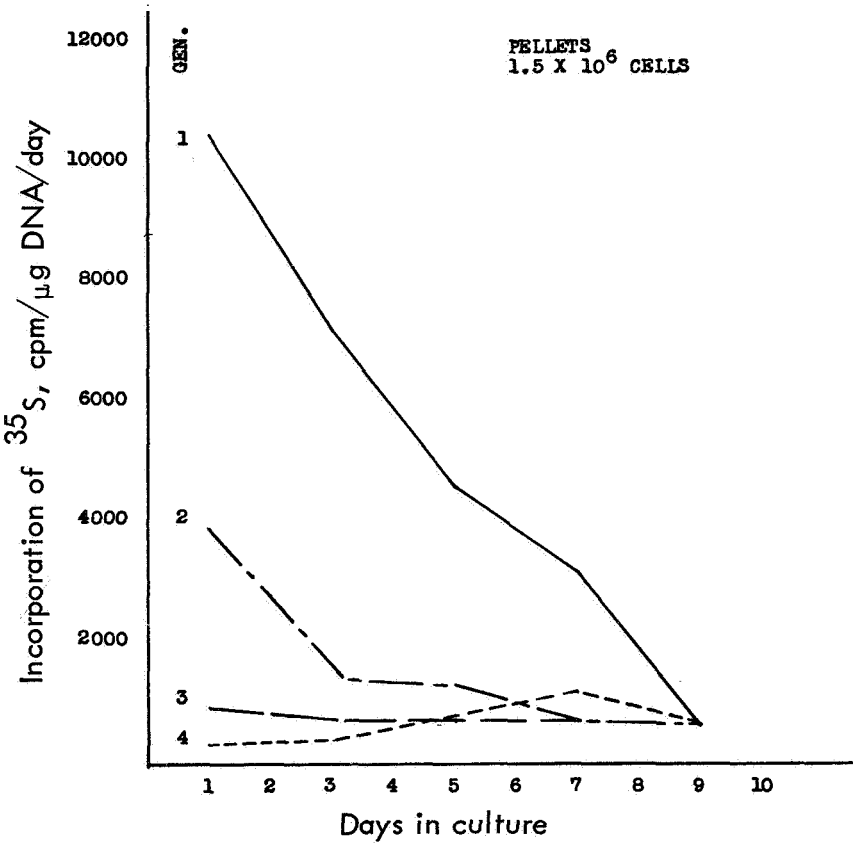

FIGURE 66. An aliquot of FLC cells was spun down into a pellet, the remainder grown as cells in monolayers. After 5 days in a monolayer situation, the cells were again divided into two groups: one grown as pellets, and the other as monolayers. After another 5 days the monolayers were again divided into two groups: pellets and monolayers. This was repeated for a third time. The values shown in this figure are for the pellets of the first, second, third, and fourth generations of cells. Clearly the amount of chondroitin sulfate made by cells in pellets varies with their previous *in vitro* history.

concurrently. For example, by definition a cell only makes DNA during S. During G–1, G–2, or M, a cell does not synthesize DNA, although clearly it is busy synthesizing other molecules. Are, in fact, all the mitochondrial enzymes, RNA's, and structural proteins made at any time in the mitotic cycle or at any time in the life history of the cell? Recently, we have shown (refs. 124 and 125) that myosin is not synthesized during S, G–2, M, or even during the first 3 to 5 hours in G–1.

Returning to pure populations of chondrocytes, we would like to know what kinds of molecules they or their progeny can make and how

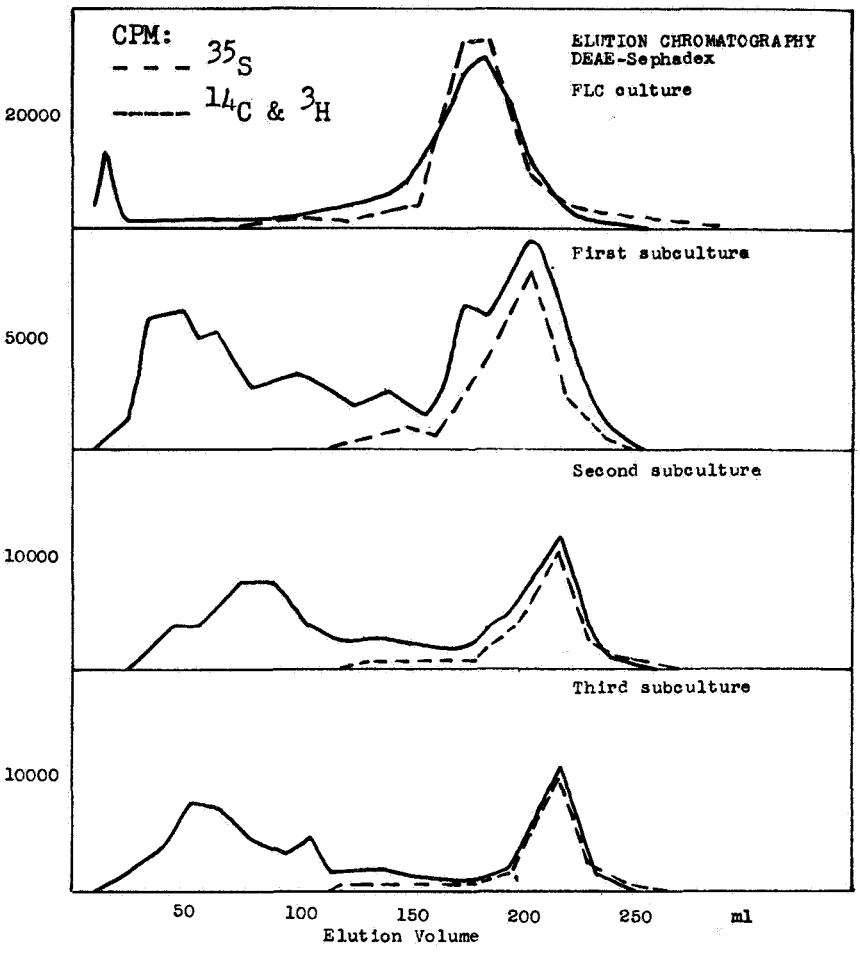

FIGURE 67. FLC cells were grown for three subcultures as monolayers and their polysaccharides analyzed by chromatography. Note that the relative amount of sulfated polysaccharide to total polysaccharide is less in the subcultured cells.

seemingly "trivial" differences in their microenvironments induce them to alter their typical products?

At this point we are not convinced that the genetic controls regulating the synthesis of, for example, amino acids or the enzymes associated with oxidative phosphorylation, are the same kind of controls that regulate the production of cell-unique molecules like chondroitin sulfate or myosin.

ROBINSON: Can you give these cells messages so that they will change——

HOLTZER: We have tried. We put them back into the animal, as you suggested, and they do not go back into the cartilage.

ROBINSON: I was thinking of the classic experiments with bacteria.

HOLTZER: No; there is no tissue system in which anybody has instructed cells to make anything. I would make that statement categorically; I said tissue cells.

NICHOLS: But you have informed these cells, because when you plated them you were placing them under a new set of conditions, if you like; you were subjecting them to a new set of stresses which was interpreted by the cells as a stimulus to do something different from what they were doing before. This is an extremely important point that we have ignored. For example, how does a bone cell know that it has been in a fracture site?

HOLTZER: That is a beautiful question.

PRITCHARD: Have you taken these cells and tried to cluster them again?

HOLTZER: Yes.

URIST: We have done a similar experiment. We have transplanted a pack of chondrocytes back into the donor, and the cells do not resume chondrogenesis.

MCLEAN: I want to throw a monkey wrench into this machinery we have been talking about. It is not mine but something that Frost (ref. 118) has published; he calls it a concept. He starts with a line of stem cells, and then some of these stem cells divide. He has introduced the idea that every time a stem cell divides, it produces an undifferentiated reserve cell and simultaneously produces one differentiated cell. For instance, a stem cell could give rise to an osteoblast and another stem cell.

PRITCHARD: I would like Dr. Young to comment on this because he has been concerned with these relationships as much as anyone else; and Dr. Owen, too.

HOWELL: Would Dr. Young also comment on whether cell death is part of this picture?

PRITCHARD: That these eventually finish their life cycle and die.

YOUNG: I think most of us who have been studying normal bone growth have seen practically no cell death. I would rather address my comments to the important point, which grew out of Dr. Holtzer's presentation and was seconded by some others, that the critical information for these various activities is already in the cell, coded in its DNA. One need not provide the cell with information-bearing macromolecules, as in the transformation experiments, to call forth from it potentialities which it might not have shown in the living organism.

I prefer to think, although we are not down to control mechanisms, that the effect of the microenvironment is among the most important

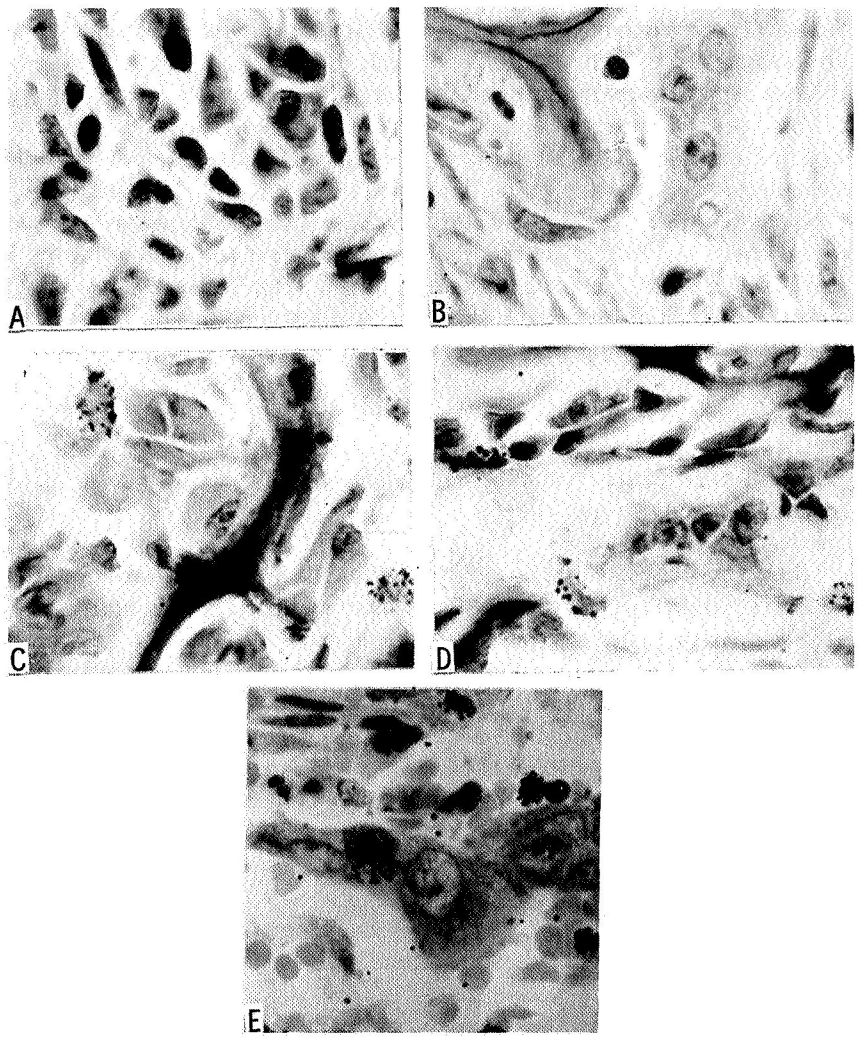

FIGURE 68. Photomicrographs of sections of tibial methaphysis of 1-week-old rats. PAS-hematoxylin stain. 920×.

(*a*) Osteoblasts and osteoprogenitors are seen in this field. Note the two osteoprogenitors in prophase near the center.

(*b*) An osteoclast and several osteoprogenitors are visible.

(*c*) One hour after injection of ^{3}H-thymidine. Several osteoprogenitors are labeled, indicating DNA synthesis prior to mitosis. Osteoblasts are unreactive. Autoradiograph.

(*d*) One hour after injection of ^{3}H-thymidine. Labeled nuclei are seen in osteoprogenitors, but not in the osteoclast (center). Autoradiograph.

(*e*) Sixteen hours after injection of ^{3}H-thymidine. Labeled nuclei are now found in osteoclasts, indicating that these cells are derived through specialization of osteoprogenitors. Autoradiograph.

factors in the control mechanism. While on this point I would like to show some figures.

One of the requirements for working with tritiated thymidine is to be able to classify the cells. Figure 68(*a*) shows the cells, the osteoblasts, that one sees around the bone. However, there are also pale-staining, rather fusiform cells, which I call osteoprogenitors.

Figure 68(*b*) is of another region and shows an osteoclast; again there are several of the pale-staining cells, the osteoprogenitors.

Work with tritiated thymidine has been repeated in many laboratories. It shows that osteoblasts and osteoclasts are incapable of reproducing themselves. They do not take up thymidine, they do not synthesize DNA, and they do not divide (refs. 126 and 127).

However, shortly after the injection of tritiated thymidine, we find that the cells which are synthesizing DNA and will divide are the pale-staining cells, the osteoprogenitors, as shown in figure 68(*c*). The specialized osteoblasts rarely take up thymidine.

Figure 68(*d*) is of another field and shows an osteoclast; again, synthesis of DNA and preparation for cell division are occurring in the pale-staining osteoprogenitors. Shortly after the completion of DNA synthesis, these cells divide. We can keep track of them because they are radioactive.

Figure 69 is a scheme of the cell cycle. We have made radioactive those cells that were in the DNA synthetic period (S) at the time of injection; and if we now, in a series of intervals thereafter, continually sample this histologically recognizable mitotic compartment (M), we will see the passage of cells from DNA synthesis into mitosis. There is a peak that represents this division, which has occurred within a few hours after DNA synthesis; some of these cells will still continue to divide (refs. 109 and 128).

This can be determined in any number of ways. One simple way is to watch them come through a second time. They are becoming more uniformly distributed temporarily throughout the cycle.

PRITCHARD: This percentage of labeled cell refers to what total population?

YOUNG: We searched the region of bone for mitotic cells and recorded the percentage of those that are radioactive.

PRITCHARD: Relative to every cell in the neighborhood?

YOUNG: No; just looking at the dividing cells. It is a percentage of all the mitoses that were radioactive, and at this interval every dividing cell is radioactive.

The point is that the labeled progenitor cells do divide. That is why they were synthesizing DNA in the first place; they were getting ready to divide. Some of them continue to divide. Others, however, change their specialization, reorganizing their metabolic machinery

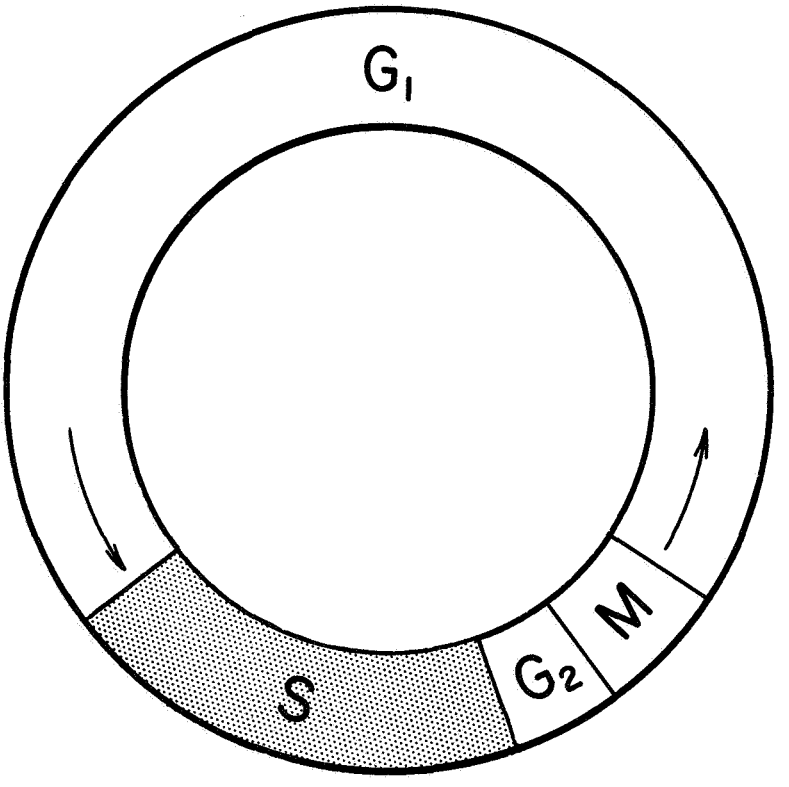

FIGURE 69. A schematic representation of the cell cycle in a dividing population. Interphase is subdivided into G_1, DNA synthesis (S), and G_2 periods. Cells engaged in DNA synthesis in the presence of ^{3}H-thymidine become radioactive. These labeled cells may then be followed through the histologically recognizable mitosis (M) phase during subsequent hours.

to become the specialized cells that we discussed previously; so that within a few hours, we can begin to detect radioactive nuclei in osteoblasts. Although the thymidine is available for only about an hour, none of these cells is initially labeled. Only after the osteoprogenitors have had time to specialize do we begin to pick up increasing numbers of labeled nuclei in osteoblasts.

Within a few hours one begins to find labeled nuclei also in osteoclasts (fig. 68(*e*)); this demonstrates that the osteoprogenitors can continue to divide or, depending upon the microenvironment in which they find themselves, may specialize as either osteoblasts or osteoclasts.

Ultimately, some of the osteoblasts become trapped in bone, in which case we then see labeled nuclei in the same cell; but now we call it an osteocyte.

I made the statement that osteoprogenitors are equally capable of specializing as osteoblasts or osteoclasts, and I would like to try to document that in several ways. (The quantitative data are given in detail by Young (ref. 128).) In young growing rat bone, the percentage of progenitor cells initially labeled is on the order of 25, 15, and 5 percent in metaphysis, endosteum, and periosteum, respectively.

If we chart the gradual appearance of labeled nuclei in osteoclasts, we find that in the metaphysis, the proportion peaks and will not exceed about 25 percent, in the endosteum it does not exceed 15 percent, and in the periosteum, 5 percent. The same analysis can be made for osteoblasts. The numbers are even more convincing. In the metaphysis the proportion peaks at about 25 percent, in the endosteum at about 15 percent, and in the periosteum, at about 5 percent.

In an effort to demonstrate that these cell specializations can be affected by the microenvironment, which is not a very daring statement, we can alter the environment one way or the other with different experimentally induced changes. In this regard, I have investigated the influence of parathyroid extract (fig. 70(*a*)). The remarkable increase in bone resorption in the treated animal is accompanied by rapid changes in cell specialization.

At the beginning of the experiment in the young rat, we see the normal picture (fig. 70(*b*)) with large numbers of osteoblasts. Within 4 or 5 hours after injection, we begin to see changes in the morphology of the cells. At 12 hours (fig. 70(*c*)), some cells can still be classified as osteoblasts, but some are beginning to assume the morphology of the progenitor cells.

A few hours later (fig. 70(*d*)) there are large numbers of osteoclasts forming by specialization from the precursor. There are essentially no osteoblasts under these high doses, but there are a large number of osteoprogenitors.

At the height of the effect of the hormone, which in these young rats is about 22 hours after injection (fig. 70(*e*)), we find large numbers of osteoprogenitors and no osteoblasts. When we provide these osteoprogenitors with tritiated glycine, we may recall how greatly glycine was concentrated on osteoblasts (see fig. 60). In contrast, these cells not only look like osteoprogenitors, they are behaving like osteoprogenitors, as shown by their diminished utilization of the labeled glycine. In fact, they are osteoprogenitors.

PRITCHARD: May I ask what are the cells along the spicule if they are not osteoblasts?

YOUNG: I think by the absence of any cytoplasmic basophilia whatsoever and by their very low utilization of glycine——

PRITCHARD: They are simply not working. They are inactive osteoblasts. Why not?

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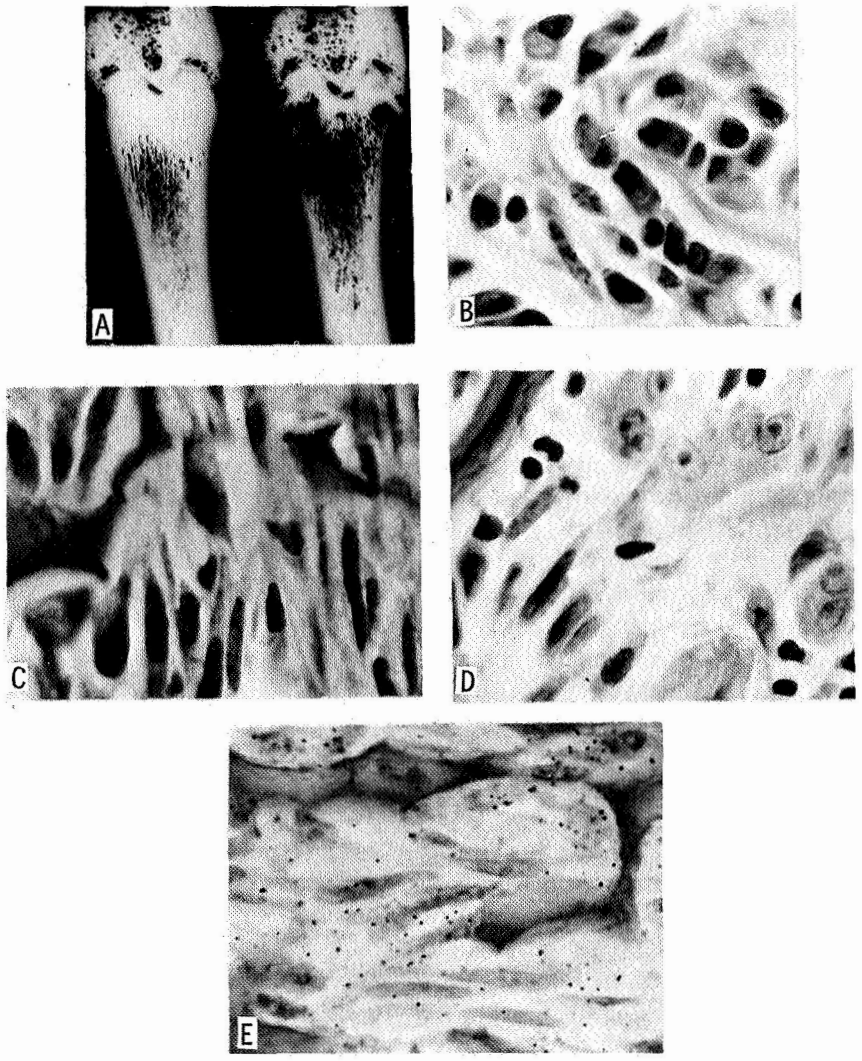

Figure 70. Effect of parathyroid extract (PTE) on rat tibias.

(*a*) Roentgenograph of tibias of 4-week-old rats. Control (left) and sacrified 48 hours after large dose of PTE (right). Note the marked resorption in metaphyseal region.

Photomicrographs of sections of tibial metaphysis of 1-week old rats. PAS-hematoxylin stain. 870×.

(*b*) Prior to onset of treatment; large numbers of osteoblasts are present.

(*c*) Twelve hours after PTE. Osteoblasts in process of returning to osteoprogenitor state under changed microenvironment.

(*d*) Eighteen hours after PTE. Most of bone cells are now specialized as either osteoprogenitors or osteoclasts.

(*e*) Twenty-two hours after PTE and 30 minutes after injection of ^{3}H-glycine. Osteoprogenitor cells, formed through respecialization of osteoblasts under the influence of PTE, fail to concentrate labeled glycine as they did in the osteoblastic state. Autoradiograph.

YOUNG: I prefer to call them osteoprogenitors. If, in the same experiment, we provide these cells with tritiated thymidine, all of these cells appear to be in the osteoprogenitor state.

Table IX is an effort to put this effect in quantitative terms. The grain-count ratio represents, in a glycine-treated animal, the ratio of silver grains over osteoblasts compared with those over osteoclasts. As I tried to demonstrate earlier, there is an enormous preponderance of glycine utilization in osteoblasts as compared with osteoclasts.

TABLE IX

PERCENTAGE OF ^{3}H-THYMIDINE-LABELED NUCLEI IN OSTEOBLASTS AND OSTEOCLASTS IN RIBS AND TIBIAS OF RAT [a]

Experimental data	Osteoblasts		Osteoclasts	
	Rib	Tibia	Rib	Tibia
Normal animals	23	16	12	10
PTE, given 1 to 7 hours after ^{3}H-thymidine injection	2	1	12	11
PTE, given 12 to 24 hours before ^{3}H-thymidine injection	26	15	1	1

[a] Averaged percentages, taken from data reported by Young (refs. 109 and 111).

Parathyroid extract is given at the beginning of the experiment. The rats are sacrificed at different intervals thereafter. As the osteoblast undergoes morphologic changes back to the osteoprogenitor state, there is a rapid decrease in this physiologic measurement of the protein synthetic activity of these cells. At about 22 hours after injection the cells look like osteoprogenitors, behave like osteoprogenitors with respect to thymidine, and behave like osteoprogenitors with respect to glycine. One is led to conclude that they are indeed osteoprogenitors.

If we allow the animals to recover, within about 2 days after the height of the effect of the parathyroid extract, the cells will have returned to their normal behavior. The osteoblasts are repairing the damage; and throughout these rapid changes in cell specialization, there is no observable increase in cell death. I think this table is an important one. The experiments pose the following two questions. Are the progenitor cells capable of specializing as osteoblasts or osteoclasts? Can we influence this specialization by changing the microenvironment of the cells? I think the answer to both of these questions is "Yes."

If the normal animal, the rat in this case, is injected with tritiated thymidine, the initially labeled cells are the osteoprogenitors. If we allow the animal to survive to a day or so after injection, we find that some of the osteoprogenitors have specialized as osteoclasts and others have specialized as osteoblasts, depending upon their location in the bone and the microenvironmental stimuli which were acting upon them.

If, instead, we follow the thymidine treatment by a large dose of parathyroid extract, we find that, a day or so later, large numbers of the initially labeled osteoprogenitors have specialized as osteoclasts. Very few osteoblasts have been formed under these conditions of high levels of parathyroid extract in the cellular environment.

In the third experiment, we first pretreat the animal with parathyroid extract to induce the osteoblasts to revert to the osteoprogenitor state. If, at that time (when the bone is filled with osteoclasts and progenitors) we provide the animal with thymidine to label the osteoprogenitors, and then allow it to recover, we find that during the recovery period the cells preferentially specialize as osteoblasts.

So, I think that these are different functional states of the same cell, and that the specialization of the progenitor cell in either one or the other direction (as well as in various additional directions which I have not had the opportunity to discuss), is determined by the immediate microenvironmental circumstances in which the cell finds itself.

PRITCHARD: There are two important concepts here. One is the reversibility of this chain of cells, and the other is the effect of parathyroid hormone on the cell population. I do not know whether Dr. Owen wants to say anything about the first question of reversibility——

OWEN: No. I think Dr. Young's experiments are very elegant.

URIST: Dr. Pritchard has made two important points this morning; one, that there is a classification problem; the other is the target cell for hormones that affect bone. Is the progenitor cell the target cell for parathyroid hormone? What is the target cell for thyrocalcitonin?

PRITCHARD: I think Dr. Talmage has some ideas on this.

TALMAGE: I have a few figures.

URIST: First, I would like to ask Dr. McLean to show his figure; there is just one.

MCLEAN: I would like to ask Dr. Young, are these experiments all on rats?

YOUNG: Yes.

MCLEAN: I would like to point out that the reaction of the rat to parathyroid hormone is different from that of any other mammal that I know anything about. It is possible with relatively low doses of parathyroid extract to produce this state of hyperostosis, to make everything differentiate into osteoblasts without ever going through

the stage of increased resorption and increased osteoclastic differentiation.

YOUNG: With repeated low doses of beef parathyroid extract, experimental animals may produce antibodies against the foreign protein, while the chronic doses may also inhibit the endogenous parathyroid secretion (ref. 129).

MCLEAN: The only thing I am saying is that your statistics are for the rat, and that represents a different cycle from what one will find in any other animal.

YOUNG: The absolute numbers of how many progenitor cells are labeled is unimportant; it does not matter. Parathyroid extract is used here only as a tool to demonstrate the interconversions of the cells, which can be demonstrated in many ways. I was not studying the effect of parathyroid extract from the standpoint that Dr. Talmage might be.

MCLEAN: My point is that this selective differentiation into osteoclasts or osteoblasts according to the time that you give the parathyroid extract is something special for the rat.

YOUNG: I do not believe that is true, Dr. McLean. The formation of increased numbers of osteoprogenitors and osteoclasts in response to a single, large dose of parathyroid extract is very striking in the young rat, but is by no means unique in this animal. For example, it has also been observed in young guinea pigs (ref. 130), mice (ref. 131), dogs (refs. 132 and 133), and pigeons (ref. 134).

The preferential formation of osteoblasts during the recovery phase after a single, large dose of parathyroid extract (refs. 111 and 135) represents a return toward the normal condition.

On the other hand, if small doses of the extract are repeatedly administered to the rat, it is indeed possible to obtain a preferential and exaggerated formation of osteoblasts without previous increase in osteoclast formation (ref. 136) or following a transient increase in the numbers of osteoclasts (refs. 129, 137 and 138). A similar phenomenon has been reported in the mouse (ref. 139), indicating that the rat is not unique in this respect either.

ROWLAND: Dr. McLean, do you have experimental evidence for other species to contradict these experiments?

MCLEAN: Yes. Years ago, when we were working very actively on parathyroid extract, we tried to reproduce these phenomena in other animals. We ran through the whole gamut of experimental animals. We never saw this hyperostosis, as we were calling it then, except in the rat.

YOUNG: I believe the significant thing about these experiments is the demonstration that these cell interconversions take place. If we set aside parathyroid extract and looked at the work that came out

of your laboratory, Dr. McLean, some of the early work on cell interconversions in birds during the egg-laying cycle, we would find these studies demonstrated precisely the same thing, the interconversion of the cells of the bone series. The osteoprogenitors were at that time called, I think, reticular cells.

MCLEAN: But that was not parathyroid extract.

YOUNG: No; it was not, but it demonstrated the same basic phenomenon of cell respecialization which to me is the most important point.

MCLEAN: I agree with that. During the experiments we did many years ago (ref. 140), we ran into a phenomenon, also peculiar to the rat, of cell death under the influence of large doses of parathyroid extract (see fig. 111).

We observed osteocytes with pyknotic, or disintegrated, nuclei in rats 12 hours after intraperitoneal injection of 1000 units of PTE, a highly toxic dose; we were quite excited about this at the time. We thought it was the mode of action of the parathyroid hormone (ref. 133). We never were able to duplicate this, however, in any other animal (ref. 135).

In animals in the same series, this effect was followed by the stage of hyperostosis, new bone building, and differentiation of large numbers of osteoprogenitor cells into osteoblasts. The first stage is osteoclastic with the peculiar phenomenon of cell death.

URIST: Thank you. Now, Dr. Talmage.

FREMONT-SMITH: You must explain why my tissue culture idea is a red herring as well as a grunion.

TALMAGE: I would like to leave it to Dr. Raisz to explain the reasons tissue cultures using bone are subject to misinterpretation, since he is an expert in this field. The primary problem is that as yet no one has been successful in getting a pure culture of one type of bone cells. Using cartilage, the situation may be different; so discussions based on tissue culture of cartilage cells may not be apropos to bone problems.

But I would like to change the subject now, and discuss the influence of parathyroid hormone on osteoclasts. Let me say at the beginning that all our work was done using rats. The fact that experts have questioned the use of rats because the bone of this species is different from that found in either man or dog actually bolsters my argument. My primary thesis is that the effect of parathyroid hormone is not on osteoclasts, but on the formation of osteoclasts. Since the rat is the only species in which one can really show this action of the hormone on osteoclasts, this bolsters, even further, my argument that calcium homeostasis is not a function of the osteoclast. However, the data which I would now like to present are a demonstration of the site of

action of the parathyroid hormone in stimulating the production of osteoclasts in the rat.

URIST: What is the target cell for parathyroid hormone?

TALMAGE: It is the mesenchymal cell. In the matter of terminology, we feel that because the term "mesenchymal cell" is an older term it should have preference; we should use the older terms unless we have good reasons for changing them. Otherwise, I do not object to Dr. Young's "progenitor" cells.

The first point is to demonstrate, what all of us must know, that in the rat there is a marked effect of parathyroid hormone on osteoclast production.

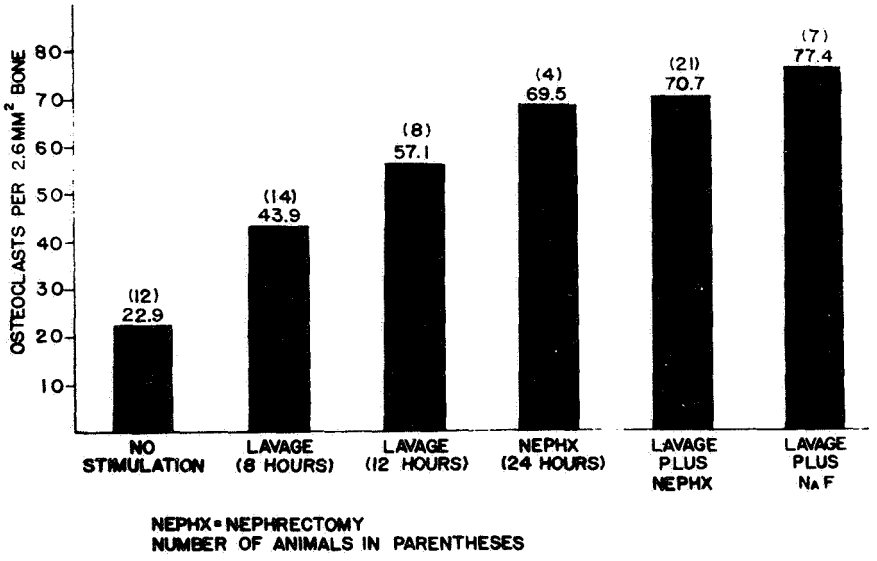

FIGURE 71. Methods of increasing osteoclast numbers in the metaphysis of the femur. [From Talmage et al. (ref. 141); reprinted by permission of the publisher.]

Figure 71 is from an earlier publication (ref. 141). It merely recalls for you the fact that one can quantitate the increase in osteoclasts in the metaphysis of the femur of the rat as a function of parathyroid activity. The normal osteoclast count is given at the left. After stimulation by peritoneal lavage with a calcium-free rinse, which is one of the best ways to increase endogenous production of parathyroid hormone, it is easy enough to show that there is a direct relation between the number of osteoclasts and endogenous parathyroid secretion.

Another method for stimulating secretion is to nephrectomize the animal. This, because of the subsequent rise in phosphate, causes a

decrease in calcium and therefore a stimulation of the animal's parathyroids. This increases the osteoclast count.

The method by which we produced the largest increase in number of osteoclasts, as the result of increasing the animal's own endogenous hormone production, was to use a calcium-free lavage containing fluoride. This fluoride produced a decrease in the solubility of the bone mineral. This resulted in an increased stimulus to the parathyroids. With this background, let us look at the source of these new osteoclasts.

TABLE X

EFFECT OF PERITONEAL LAVAGE ON PERCENT NUCLEI LABELED 48 HOURS AFTER ^{3}H-THYMIDINE INJECTION

[Adapted from Talmage et al. (ref. 141)]

Group	Osteoclasts	Osteoblasts
Normal animals	10.6 ± 0.48	20.2 ± 1.01
Normal, lavaged	1.8 ± 0.42	18.0 ± 0.68
PTX animals [a]	9.8 ± 0.83	20.8 ± 0.66
PTX, lavaged [a]	11.3 ± 0.44	19.3 ± 0.68
Average of all groups: Osteoblast: 18.8 ± 0.91 Mesenchymal cells: 6.3 ± 0.48		

[a] PTX = parathyroidectomy.

The data in table X are based on Dr. Young's work. Dr. Young just demonstrated that 48 hours after tritiated thymidine injection, the labeling of mesenchymal cells goes from a high of about 25 to 30 percent back down to 6 percent. Table X indicates that our data substantiate this. Also, by that time, the percent of labeled osteoblasts is up to 18 percent, and the osteoclasts up to 10 percent. These figures agree with those given by Young.

Now, if 48 hours after ^{3}H-thymidine injection, we produced a marked increase in the number of osteoclasts by an 8-hour peritoneal lavage, we should be able to determine if the osteoclasts were derived from osteoblasts. If so, the percentage of nuclei labeled would have to end up somewhere between the starting percentage for the two types of cells. If they were to come directly from mesenchymal cells alone, one would expect labeling to be between 10 and 6 percent. But you will see that the number of osteoclasts that were labeled after this sudden burst of production of osteoclasts dropped below both that for the original osteoblasts and for the mesenchymal cells (ref. 141).

This threw us into a quandary, as we had been hoping to show that these new osteoclasts came from mesenchymal cells. However, if one assumed that most of the mesenchyme cells that produced new osteoclasts had to first go through mitosis, this would dilute the label sufficiently so that the radioactivity of thymidine would not be detectable in the osteoclasts.

While we had eliminated the osteoblast, we did not feel we had eliminated mesenchyme cells, so we shifted, then, to mesenchyme cells and a study of the uptake of tritiated cytidine.

TABLE XI

EFFECT OF PARATHYROID STIMULATION ON PERCENT NUCLEI LABELED 30 MINUTES AFTER ^{3}H-CYTIDINE INJECTION

[Adapted from Talmage et al. (ref. 141)]

Group	Number	Mesenchyme cells
A. Nonlavaged: Intact	7	10.2 ± 0.69
B. Lavaged:		
16 hours, intact	7	18.2 ± 0.70
8 hours, intact	8	20.2 ± 0.69
16 hours, PTX [a]	7	11.5 ± 0.87
All groups	16	
Osteoblasts: 9.6 ± 0.59		
Osteoclasts: 4.1 ± 0.49		
Osteocytes: 1.2 ± 0.33		

[a] PTX = parathyroidectomized after the eighth hour.

Table XI demonstrates the labeling of mesenchyme cells with tritiated cytidine one-half hour after injection (ref. 141). We used the ½-hour period because at that time the cytidine is still in the nucleus. Examination of these data demonstrates that the stimulus for increasing endogenous parathyroid hormone secretion produced by the peritoneal lavage technique caused a doubling of the percentage of mesenchyme cells labeled. This could be inhibited by parathyroidectomy. At no time could we see any effect on the labeling of other cells; that is, the osteoblasts, the osteoclasts, or the osteocytes. Therefore, we assume that parathyroid hormone must have been affecting the RNA turnover in mesenchyme cells.

PRITCHARD: You did not have any difficulty in deciding which was a mesenchyme cell and which was not?

TALMAGE: I am sure we have the same difficulty that everybody else has. In our study we call mesenchyme cells the large group of

cells that are not lying against the bone, are mononucleated, and somewhat spindle shaped.

PRITCHARD: If it is not an osteoblast or an osteoclast, it is a mesenchyme cell.

TALMAGE: Since we are amateur histologists, if it is not an osteoclast or an osteoblast, and if it is in an area away from the bone, we usually call it a mesenchyme cell. This is a very broad term which includes all stages in the development of the cell.

PRITCHARD: You would not have counted those in the endothelium?

TALMAGE: No.

PRITCHARD: You would not have counted any hemocytoblasts you picked up?

TALMAGE: We tried not to. Therefore, we feel that we have demonstrated that what we call mesenchyme cells are the cells affected by parathyroid activity.

TABLE XII

EFFECT OF ACTINOMYCIN D ON OSTEOCLAST COUNT IN FEMORAL METAPHYSIS OF PERITONEAL LAVAGED RATS

[Adapted from Talmage et al. (ref. 142)]

Group	Count
Controls, nonlavaged (12)	23.7 ± 1.2
Controls, lavaged 8 hours (10)	53.8 ± 1.7
PTX, lavaged 8 hours (6)	32.6 ± 1.6
AMD, injected 8 hours before lavage (6)	37.1 ± 4.2
AMD, added to lavage rinse (6)	56.6 ± 4.0

Numbers in parentheses indicate number of animals.

The data in table XII back up this assumption (ref. 142). In our lavage system, when we added actinomycin D to the lavage fluid, it caused a fall in the rate of calcium removal by the fourth or fifth hour. The important point here is that adding the drug to the fluid did not prevent the normal increase in osteoclasts which occurs with lavage. Normal increase is between 50 to 100 percent in 8 hours. When, however, the drug is given 8 hours prior to the start of the lavage, it prevents this increase in the number of osteoclasts formed.

This suggested to us that whatever parathyroid did, it did immediately, before actinomycin D could knock out RNA production. Despite the presence of the drug, the simultaneous stimulation by endogenous hormone was still able to produce osteoclasts. I think this is a very important consideration, as it indicates the rapidity of the action of the hormone.

TABLE XIII

EFFECT OF ^{239}Pu ON OSTEOCLAST COUNT AND ^{3}H-THYMIDINE-LABELED MESENCHYME CELLS IN RAT FEMORAL METAPHYSIS

[From Doty et al. (ref. 143)]

Group	Number of animals	Osteoclasts per field	Number of animals	Mesenchyme cells ^{3}H-thymidine-labeled, percent
Control animals.....	11	33.0±1.5	3	19.2±0.6
^{239}Pu, 24 hours.....	7	35.4±2.3	3	[a] 12.0±0.5
Control, lavaged.....	14	54.1±2.6	.....	.....
^{239}Pu, lavaged.....	12	[b] 41.9±1.6	.....	.....

[a] Significantly lower than noninjected control animals ($p < 0.001$).
[b] Significantly lower than noninjected lavaged animals ($p < 0.001$).

Table XIII demonstrates the effects of plutonium (ref. 143). We had discovered that if plutonium is injected into an animal, the first effect produced in 24 hours——

BAUER: How much?

TALMAGE: The dose was ample, 1 mg/100 g animal weight. I am not trying to stress long-range effects of plutonium. An immediate effect of plutonium is that by 24 hours the bone was unable to respond to the stimulus of endogenous parathyroid secretion, at least in that no osteoclasts were found following peritoneal lavage. This is illustrated in table XIII. Also summarized are data on the inhibition by plutonium, under these conditions, of the uptake of ^{3}H-thymidine by mesenchyme cells. These data would again indicate that the effect of parathyroid hormone is on mesenchyme cells.

I would like to discuss figure 46 in more detail and with a different emphasis. It represents some of our recent work and is preliminary data.

In these experiments the rats were subjected to peritoneal lavage for up to 8 hours. Following lavage, the bones were dissected out as fast as possible. It was only 8 minutes from the time the animal was killed until the bone was placed into the incubation flask. Tritiated cytidine and thymidine were added to the serum and incubated with the bones for 1 hour. RNA and DNA were extracted and the specific activities were determined.

I would like to emphasize the point that the parathyroid stimulation needed to last only 20 minutes to produce a very marked increase in the uptake of ^{3}H-cytidine by RNA. This increase was seen only in

the metaphysis during the early hours, but later during the lavage both the metaphysis and the diaphysis were affected.

The fact that the early stimulation is in the metaphysis suggests that we are seeing effects primarily in the large population of mesenchyme cells which are located in this area of bone.

I would like to use figure 46 to demonstrate one other point which we have not yet proved statistically but which may become important. In this figure, the ^{3}H-cytidine uptake by the two types of bone in control rats is shown as the baseline in each graph. The value for the nonlavaged animal, parathryoidectomized for 18 hours, is given at the zero hour on the lavage time schedule. In the metaphysis, parathyroidectomy alone increased cytidine incorporation into RNA. There was no further effect due to lavage. This is the first time that we have been able, with our technique, to demonstrate a possible effect of endogenous parathyroid hormone on what I assume must be osteoblast function. It appears that parathyroidectomy released a suppressor of RNA synthesis. Following stimulation by peritoneal lavage, the stimulation for RNA production in the mesenchyme-cell population reversed this suppression and ^{3}H-cytidine uptake increased. This, of course, is speculation.

The last point that I would like to emphasize is that there is a marked difference between the effects in the metaphysis and those in the diaphysis. This was mentioned briefly in Session I. If we are correct in assuming, as we have indicated before, that the calcium homeostatic function is centered primarily in the areas of the diaphysis, we would interpret the data in this figure to indicate that the effects on RNA production here is not a mechanism by which certain bone cells are controlling calcium homeostasis, but rather a parathyroid stimulation is causing the cell to change its orientation, the forming of new osteoclasts.

Figure 47 illustrates ^{3}H-thymidine uptake into DNA; it is merely to demonstrate that DNA is affected by the same system. Thymidine uptake is also affected, but there is a time delay. It suggests that the parathyroid stimulus increases the mitotic rate in the mesenchyme population, causing reorientation of a certain percentage of these cells into osteoclasts.

To summarize, we believe that the stimulus of parathyroid hormone is not on the osteoclast itself, but on the mesenchyme cell population to produce more osteoclasts and that these latter cells are probably not concerned with the control of calcium homeostasis.

URIST: Thank you very much. Dr. Pritchard, I believe we have reached the point where it is clear—at least it is to me—that once a cell is differentiated as an osteoprogenitor cell, it can respond to hormones in various ways. Now can we discuss the subject of what

induces cells to differentiate into an osteoprogenitor cell; i.e., to get on Young's wheel?

PRITCHARD: I do not think we should take Young's wheel for granted. We have a population in which some cells are dividing, some are differentiating, and others are differentiated and actively engaging in matrix formation or destruction.

There is still doubt as to the reversibility of this system. I would like to see some concrete evidence that osteoclasts actually break up into progenitor cells.

There are many factors that can affect this cell system. I think Dr. Peck has information about a vitamin effect on the system and also Dr. Budy, about the effect of estrogen on bone.

RAISZ: Dr. Peck and I have been discussing the issue of tissue culture versus studies on tissue *in vivo*. And since the question has been raised and not answered, I think in fairness to the various groups around the table who are using different systems *in vivo* and *in vitro*, we ought to attempt to clarify what these different approaches can achieve. In a conference on topics as complex as this, we could become further confused by going back and forth between *in vivo* and *in vitro* data.

We are going to hear about what I regard as elegant *in vitro* data from Dr. Peck. This work proves something important about the way living cells work, but it does not prove anything about the way the precursors of these cells function *in vivo*. Dr. Fremont-Smith asked us to look at tissue culture to find answers to the questions that Dr. Young is raising. I would say that this was a wrong approach, because Dr. Young was asking an entirely descriptive question about the way in which a living system showed modulation of cells in response to external agents. I do not see how any *in vitro* system can tell you what the sequence of this cellular modulation would be. On the other hand, we can do much better experiments on the biochemistry of cell transformation *in vitro*.

FREMONT-SMITH: The point I wished to make is that I believe tissue culture throws light on how cells function somewhere else. From this, one can make inferences that make it possible to understand better how cells function in their normal environment.

RAISZ: Certainly, as long as you do not try to slide the data over into the *in vitro* situation.

FREMONT-SMITH: There also has been a tendency to say nothing can be learned *in vivo*. I would like to make a prediction, and I would be willing to put it on the record. But 5 or 7 years from now, there will be new information from tissue culture which will have influenced our understanding of cells *in vivo*.

BAUER: What is so special about tissue culture? Is it not true of all techniques, that one must simplify and that one must take into account a system which one is not studying? What is the difference between tissue culture and cytoanalysis? One has to introduce modification in all techniques.

FREMONT-SMITH: These are all model systems, are they not? And none of our histology tells us what the living cell is, because all of the cells are dead. I think it is worth reminding those who are embedded in histology, which is an essential feature of our understanding, that they are not looking at living cells; they are making implications. The only place one can see living cells is in tissue culture, and these cells are not the same; they are in a different environment from those *in vivo*. It is this interaction balance, however, which I am sure will throw light on many fields.

I think that the reaction one gets—I have seen it again and again in these conferences over 30 years—is a great reluctance to accept data coming from another technique or another field. Even the introduction of a new stain is very disturbing to histologists at times, until it becomes more standard. What I am working for, and one of the main purposes of our conference, is to see what we can each learn from the other's techniques, not what we can throw out.

TALMAGE: If one works with an isolated system such as tissue culture, the tendency is to explain the entire physiologic process on the basis of the results from the isolated system. In my opinion, one must make a sincere effort to study the entire physiologic process in the intact animal, and be very cautious of those results from isolated *in vitro* systems which appear contradictory or are unexplainable in relation to the physiologic process as seen *in vivo*.

NICHOLS: Let us go on to Dr. Peck, Mr. Chairman.

PRITCHARD: The problem is simply to find out how cells behave in different environments, both *in vitro* and *in vivo*.

PECK: We have been working on a system that has certain similarities to Dr. Holtzer's. We have been able to disperse cells from fetal and newborn rat calvaria using a crude collagenase preparation—incidentally, we cannot do it with trypsin—and have studied the cells from a number of standpoints; most recently, collagen synthesis (refs. 144 and 145). We made no claims that the cells we have isolated are bone cells, for they have not been shown to produce bone *in vitro*, and it is quite likely that we have harvested a heterogeneous cell population. We have been primarily interested in the relationship between cell proliferation, cell density, and responsiveness to humoral agents, in particular, ascorbic acid and parathyroid hormone.

If we suspend cells in simple incubation medium, we can demonstrate

synthesis of a hydroxyproline-containing protein. The ability of these freshly isolated cells to form peptide-bound hydroxyproline is indicated in figure 72, which depicts the incorporation of radioactive proline into protein, and the appearance of radioactive hydroxyproline in protein, after incubation of cells with labeled proline. The appearance of hydroxyproline, which we can equate with collagen synthesis, increases during the first 6 hours of incubation. After 12 hours, no further hydroxyproline is formed.

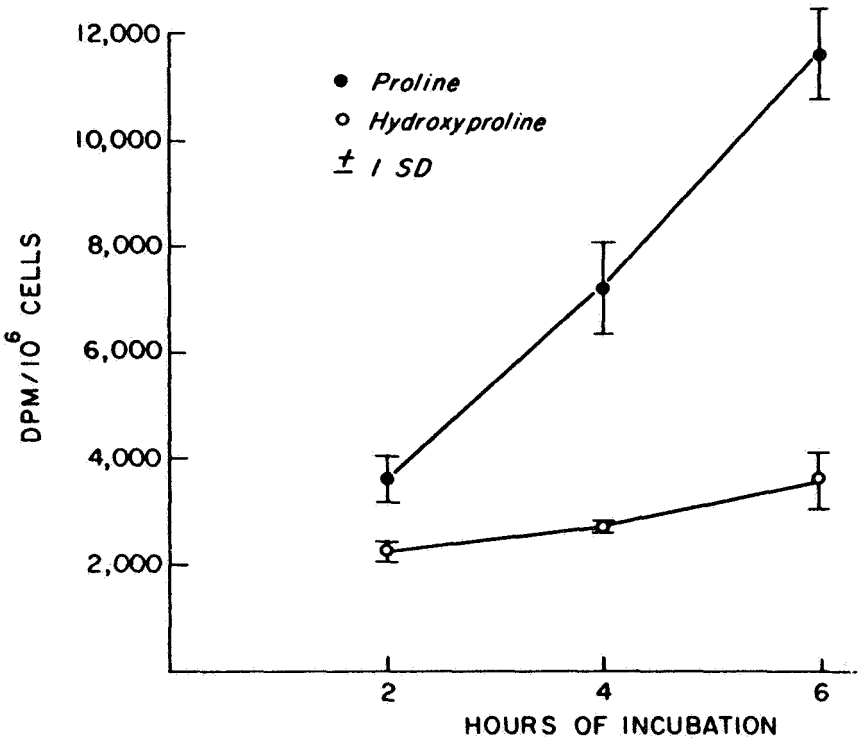

FIGURE 72. Collagen synthesis of freshly isolated bone cells. Freshly isolated cells were incubated in Krebs' Ringer bicarbonate buffer, pH 7.4, gas phase 95 percent O_2, 5 percent CO_2, containing L-proline-U-^{14}C, dialyzed bovine serum albumin 0.5 percent, glucose 0.11 *M*, and penicillin and streptomycin 100 units each per milliliter. Each point represents the mean of three flasks. [From ref. 145; reprinted by permission of the publisher.]

FREMONT-SMITH: These are all rat bone?

PECK: These are from the calvaria of rat fetuses that have been dispersed by collagenase. Collagen synthesis by freshly isolated, suspended cells does not respond to a variety of humoral agents,

including insulin, parathyroid hormone, growth hormone and, most disturbingly, ascorbic acid. As you know, ascorbic acid has been found to stimulate collagen formation in a host of *in vitro* and *in vivo* studies with many types of connective tissues. Because of this lack of responsiveness, we decided to maintain the cells under different circumstances.

We dispersed the cells in cell culture on a flat surface, apparently in a fashion similar to the system used by Dr. Holtzer. The cells then go through a period of proliferation. Figure 73 indicates the amount of DNA in the culture with respect to days of culture. It may be seen to increase modestly.

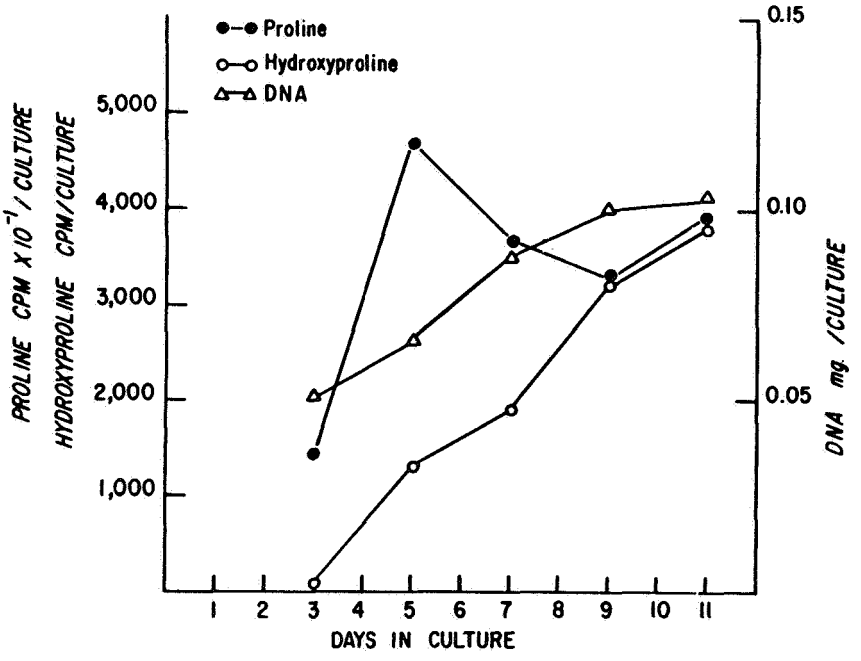

FIGURE 73. Relationships between cell proliferation and appearance of proline and hydroxyproline radioactivity in protein during culture of bone cells on a flat surface. L-proline-U-^{14}C was added to each culture 12 hours before termination, hence data indicate radioactivity incorporated during the preceding 12 hours. [From ref. 145; reprinted by permission of the publisher.]

In this particular series we plated many cells to start with. If one begins with a lot of cells, they do not divide as many times as when one starts with fewer cells. This is because proliferation is limited by the area that contains the cells. Once the cells form a layer that fills the containing area, proliferation slows markedly.

The important point in figure 73 is the appearance of collagen which is represented by the appearance of radioactive hydroxyproline in protein. This was determined by adding ^{14}C-proline 6 hours before harvesting the culture, so that each point represents the collagen that has been synthesized during the preceding 6 hours. You can see that the rate of collagen synthesis increases with the duration of culture, increases with the accumulation of DNA, and increases with increasing density of the cell population.

If you add ascorbic acid to this cell-culture system, you get a dramatic stimulation of the formation of hydroxyproline-containing protein (fig. 74). We call it collagen formation, at least in the biochemical sense. This is a dose-related phenomenon, as indicated by plotting on semilog paper the concentration of ascorbic acid in μg/ml against the appearance of hydroxyproline radioactivity expressed as μg of DNA in the culture.

URIST: Is the collagenous material uncalcified?

PECK: Yes. We have seen no evidence of bone formation in our

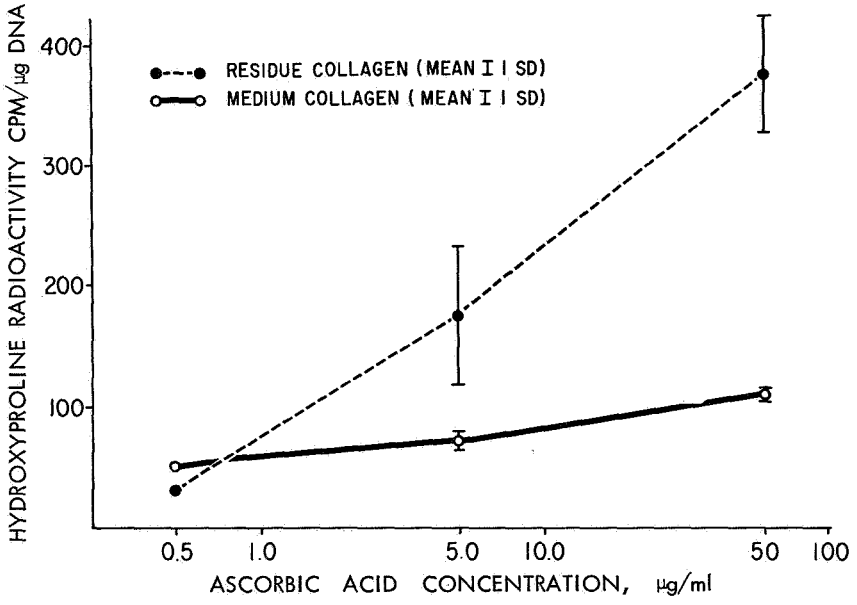

FIGURE 74. Effect of concentration of ascorbic acid on collagen synthesis by bone cells in primary culture. Cultures were incubated in minimal essential medium (Eagle) for 6 hours, in the presence of L-proline-U-^{14}C. Residual collagen reflects the hydroxyproline radioactivity of hydrolyzed collagen. Protein was isolated from the medium by Sephadex filtration before hydrolysis. Each point represents the mean of two or three flasks.

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cultures. We have seen precipitation of amorphous material, the nature of which remains to be clarified.

URIST: Have you transplanted the tissue back into a living animal?

PECK: We have placed cells in Millipore chambers and implanted them intraperitoneally in growing rats. We get what appears to be, in the Millipore chambers, amorphous material. I discussed this material with Dr. Lent Johnson at the last Gordon Conference.

URIST: Was it calcified?

PECK: Yes. However, none of us could figure out what it was. To reiterate, there is a linear relationship between the log of the concentration of ascorbic acid and the appearance of hydroxyproline (fig. 74). Note that the lower extremity of this curve represents a concentration of 0.5 μg/ml of ascorbic acid. Generally, the physiologic range of serum ascorbic acid concentrations in humans is about 25 to 30 μg/ml.

We were interested in the relationship of this ascorbic acid effect to the duration of treatment. As figure 75 indicates, we can detect changes with respect to stimulation of collagen formation within 30 minutes of the addition of ascorbic acid.

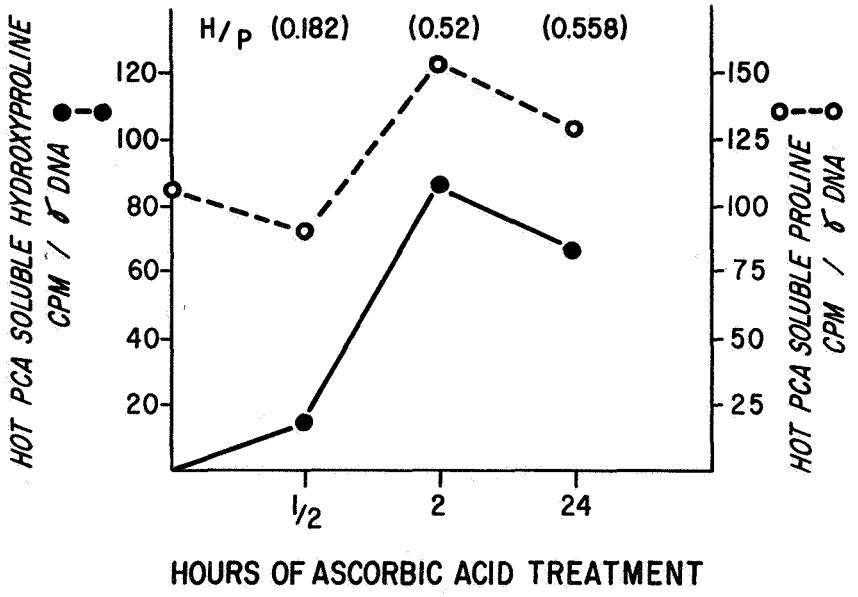

FIGURE 75. Effect of duration of exposure to ascorbic acid on collagen synthesis by bone cells in primary culture; L-proline-U-^{14}C was added to each flask 30 minutes before the end of the experiment. Collagen was isolated from the cell layers by two 15-minute extractions with 5 percent perchloric acid at 70° C.

PRITCHARD: I think one could make the point here that ascorbic acid acts on the cell and presumably acts through the DNA–RNA mechanism.

PECK: Initially, it probably acts directly on the process of proline hydroxylation. I would like to think that the stability of the collagen molecule is governed by the hydroxylation of some proline residues and that, in the absence of ascorbic acid, hydroxyproline-poor collagen accumulates. These changes can occur without postulating any fundamental change in the mechanism of the protein synthesis.

I wanted to go into this partly because of the preceding discussion about the value of *in vitro* systems. *In vitro* systems are uniquely suited to deriving strictly biochemical information in a highly controlled environment. This particular system may provide us with some information which will be of value with respect to what is going on *in vivo,* but to draw that conclusion at this point would be totally erroneous.

I will say, in addition, that cultured cells will respond to parathyroid hormone *in vitro* with an inhibition of collagen synthesis which can be reversed by ascorbic acid. At present we are studying growth hormone to see if these cells will respond to that as well.

PRITCHARD: You have a wonderful system which has already given much valuable information.

NICHOLS: Dr. Peck has brought up several important things. One is to point out what happens to cells when they are removed from their site of origin. I wonder, Dr. Peck, if you could tell us about the comparative activity of cells when they are in their normal habitat on surfaces of calvaria, and when they are in suspension?

Modification of all behavior by modification of physical surroundings is extremely important and may well be the control which lies behind differentiation. Incidentally, our *in vitro* systems are really useful here because with them we can examine directly how cells respond to such stimuli.

PECK: One thing we might have done which would have been very helpful was to do essentially what Dr. Holtzer and his colleagues did when they incubated cartilage cells in a button (ref. 146). Our freshly isolated cells were incubated in suspension. We shook them fairly vigorously. It would be interesting to see if these cells maintained more vigorous collagen formation if they were maintained in a button rather than suspended in a simple medium.

The point which remains is obviously a critical one. These cells are derived from tissues that synthesize collagen at a fantastic rate; although we have not done too many studies relating the ability of cells to synthesize collagen with respect to the amount of DNA that

has been released from the bone, our data would at least suggest that the cells are markedly impaired in their ability to synthesize collagen by the process of isolation. This may be open to question, but I think the evidence is quite clear that these are altered cells or that at least some of the cells within the population we isolated are changed.

PRITCHARD: There is another important topic we have not touched yet, the question of induction. I wonder if Dr. Saxén would like to say something about induction systems.

SAXÉN: I will briefly summarize the present situation in the field of embryonic induction and outline our way of thinking. In doing so, there will be no time to present the experimental evidence on which my summary is based, but I will say that most of it is derived from experiments with soft tissues. However, I hope that the general scheme I am going to formulate will be applicable to bone induction and osteogenesis as well, and that both Dr. Holtzer and Dr. Urist will comment on this aspect later.

In my scheme (fig. 76), I have divided the differentiation of a hypothetical cell population into different steps. The responding tissue (which is shaded in the figure) receives an inductive stimulus from the inductor tissue at the beginning of the chain of differentiative events. I do not intend to discuss the specificity of this stimulus, although I may say that I have certain doubts about its specific nature. Let us just call it a stimulus or trigger which initiates the differentiative process in our cell population. If the responding cells are to be capable

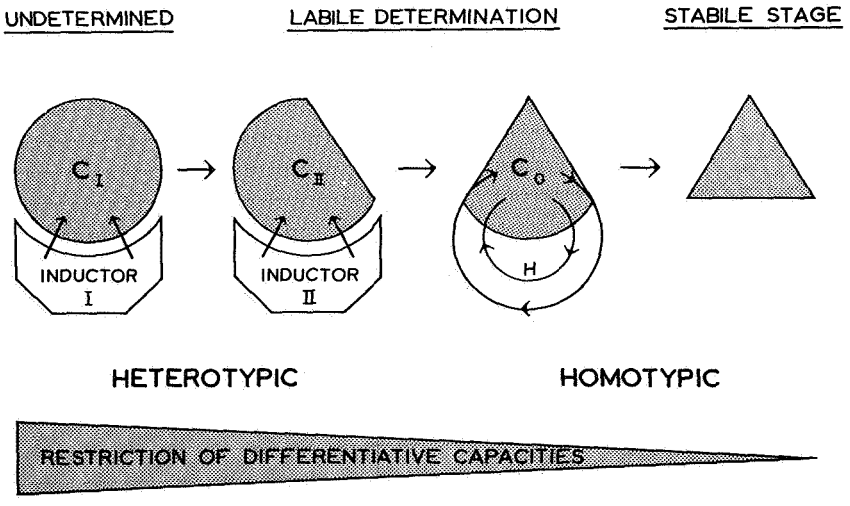

FIGURE 76. Differentiation of a hypothetical cell population.

of responding to this inductive stimulus (I), they should be competent (C_I)—a characteristic known to disappear during subsequent development.

As a result of this initial inductive stimulus, the responding cells become determined in a certain direction, and consequently lose their competence to respond to similar triggering stimuli. However, this stage of labile determination is followed by subsequent interactive processes. In some model systems at least, the primary stimulus is known to be followed by a second heterotypic induction (II), leading to subsequent determination of the cells. The cells at this stage now display another competence (C_{II}), making them capable of responding to the second inductive stimulus.

Subsequent to this (and perhaps even after several heterotypic actions), the responding cells become independent of such epigenetic stimuli, and develop autonomously.

URIST: Please define the term "epigenetic."

SAXÉN: It means "not genetically controlled."

URIST: Thank you.

PRITCHARD: Perhaps I can sum up the discussion so far as follows. From the chemical standpoint, cell differentiation implies change in the kinds or amounts of a cell's synthetic activities. Sooner or later these changes are reflected in the histologic picture—such as mitotic figures, changes of cell shape, size, structure, mobility, change in intercellular matrices, changes in the pattern and organization of the tissues composed of such cells and their matrices.

It is postulated that these changes are brought about by a series of inducing stimuli which evoke responses from competent cells.

The important questions are as follows: What is the nature of the inducing stimuli; where and how do such stimuli act on the cell; how do they select one response, or a particular set of responses, from the cell's repertoire; what is competence in terms of DNA activity; how permanent are the changes induced; what happens when a cell loses one competence and gains another competence?

Inducing stimuli are of many kinds, but now we are particularly interested in the postulated close-range ones which act between one cell and another in its immediate range, which may be like it, or quite different.

As I said, I do not know whether all of these are pertinent to bone induction or morphogenesis, and that is why I would very much like to have comments by Dr. Holtzer and Dr. Urist, who have been studying these problems.

HOLTZER: I think I have spoken enough. I thought that was an excellent summary of the problem.

PRITCHARD: There are still one or two matters to be considered. Some of these concepts are called by different names in the literature; e.g., dependent differentiation, self-differentiation, autoinduction, heteroinduction. Also, there is the question of reversibility, whether one can dedetermine, dedifferentiate.

HOLTZER: No; I disagree. I do not think we should wander all over using such words as they apply to different kinds of cells. I think we should ask Dr. Young or Dr. Peck questions, such as "When, in the life history of bone cells, do they first start making collagen?" or "Do they make collagen during mitosis, G–1, S, or G–2?" and so forth.

YOUNG: I do not believe it is made during S, when the cell is devoted to duplicating its DNA, and I do not think it is made during mitosis. I am sure it is made by osteoblasts during G–1.

PRITCHARD: It must be made by progenitor cells in some stage of their life history.

PECK: Now we are running into the problem we discussed previously of when to call collagen *collagen.* If one waits for morphologically identifiable collagen to be formed, the chances are that the dividing cell will no longer be able to make it. The question is, If there is an arrest in collagen synthesis, where does it occur? Is it arrested at an early stage of synthesis, for example, at the stage of proline hydroxylation, or is it a total arrest, presumably at the genetic level?

YOUNG: My personal bias is that changes in cell specialization are mediated by the selective activation and repression of integrated groups of genes, but I do not think we should go into this now. I would like to hear what Dr. Urist has to say.

OWEN: On the periosteal surface of the shaft of the femur of young rabbits, the osteoblasts line the surface of the bone and behind them are the preosteoblasts, their precursors. In this system, the uptake of glycine per cell in the precursors is about one-tenth the uptake in the osteoblasts.

PRITCHARD: That is a very pertinent observation.

NICHOLS: Dr. Howard Green has some evidence from tissue culture in another system which bears on this point of dividing cells (ref. 147).

HOLTZER: Let us get together. A culture, in which some cells are dividing and some are not, is not a system which permits one to say whether or not a single dividing cell is making collagen. A cell in S is not doing the same thing that a cell in M is doing.

PRITCHARD: This is an academic matter.

HOLTZER: On the contrary, I think these are the only kinds of questions we can approach, otherwise we get back to defining cells by names. You wanted a definition of differentiation, what it means, and of reversibility, and so forth——

PRITCHARD: Call it the collagenoblast or mucopolysaccharidoblast, or something like that.

HOLTZER: Names do not mean very much. We would like to know what a given cell does, and when and under what conditions it does it. We know that during M very little protein and very little RNA is made. What is the nature of such controls? What reactivates transcription and translation after each mitosis? One of the most central problems in all biology is what happens to the cell's synthetic machinery so that it appears to shut down every time the cell goes through mitosis only to be activated in the following G–1. More information on this issue might give us a better basis for words, differentiation, dedifferentiation, induction, and so forth.

PRITCHARD: Perhaps we can leave this topic for the moment and call on Dr. Urist to talk about bone induction.

URIST: The question is, What induces a cell to differentiate and to become an osteoblast? We do not know whether the tissue that Dr. Peck isolated, or the material that Dr. Holtzer explanted, contained induced cells. In the past we have assumed that a cell had been induced at an earlier stage of its development, if after transplantation it demonstrated its capacity to differentiate into an osteoblast. The conditions in tissue culture are less than adequate for osteogenesis, but in the anterior chamber of the eye, conditions are optimum. I will demonstrate osteogenesis induced by germinal cells of articular cartilage with the following figures to be discussed by Dr. Saxén and Dr. Holtzer.

When the cell is at the stage of development of an osteoprogenitor cell, it may be the differentiated form. We cannot tell the difference between a cell that is an osteoprogenitor and one that is a fibroprogenitor by morphologic criteria. We can get some idea by its location and by its rate of mitotic division. We would like to be able to identify an osteoprogenitor cell by its ultrastructure, but Dr. Robinson says that is not yet possible. We do know that because of its proximity to bone, it may have a strong tendency to differentiate into an osteoblast.

The young cells in the germinal layer of the articular cartilage (epiphyseal side) take up tritiated thymidine in larger amounts than other cartilage cells. When a thin slice of the surface of the articular cartilage, a slice containing the flattened chondrocytes, is transplanted in the anterior chamber of the eye as an isograft or as an autograft, the product is either fibrous tissue or induction of new hyaline cartilage (fig. 77(*a*)).

Another slice (fig. 77(*b*)), containing the deep or germinal layer of cells, induces bone formation. Before transplantation to the eye, the cartilage cells of the germinal layer were labeled with ^{3}H-thymidine, and the cartilage matrix was labeled with ^{3}H-glycine by intra-articular

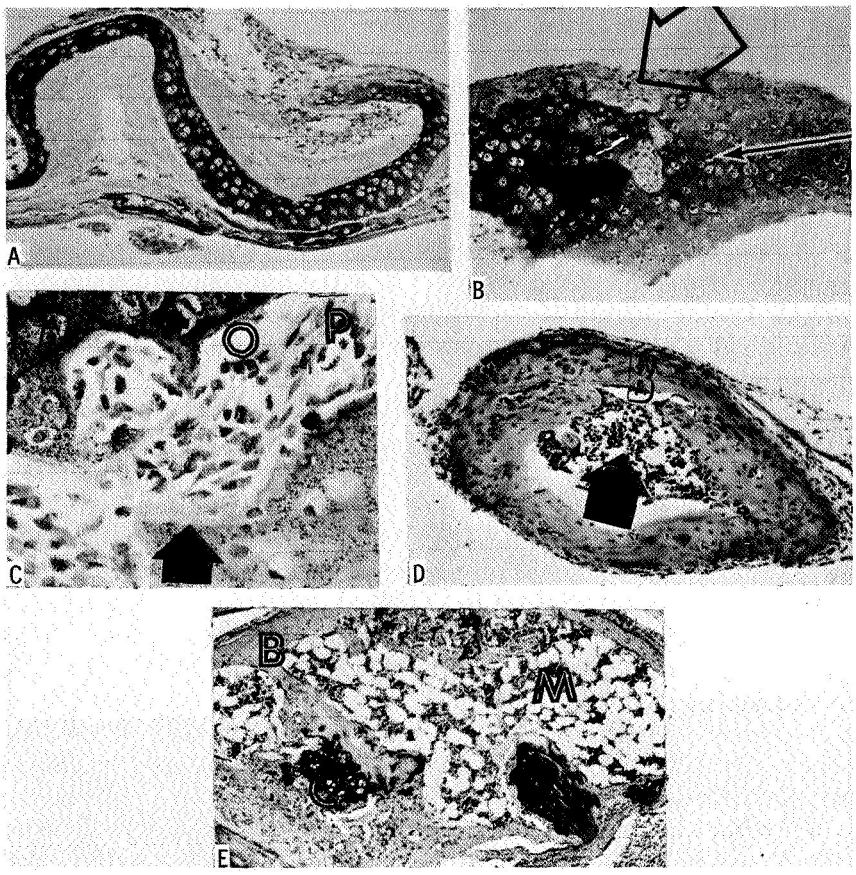

FIGURE 77. Photomicrographs of sections of articular cartilage transferred to the anterior chamber of the eye. PAS-hematoxylin stain.

(*a*) Thin slice of gliding surface of cartilage, 6 weeks in the eye. Transplant is enveloped in capsule of fibrous connective tissue; neither excavation chamber nor esteogenesis is produced. 30×.

(*b*) Coronal section, full thickness slice of cartilage: labeled chondrocytes (black and white arrow); excavation chamber filled with capillary sprouts, new connective tissue, and a pair of labeled chondrocytes liberated from their capsules and free in center (open arrow); excavation chamber filled with new cells but no labeled chondrocytes (solid arrow). 30×.

(*c*) Autoradiograph: cement line dividing glycine-labeled old cartilage from layer of unlabeled new bone matrix (solid arrow); labeled chondrocytes (open arrow); labeled progenitor cells (P) and osteoblasts (O) suggest that labeled chondrocytes modulate into bone cells. 30×.

(*d*) Ossicle formed from transplant. New lamellar bone (B) surrounds pool of marrow (M) and contains islands of unresorbed cartilage (C). 60×.

(*e*) Complete replacement of donor tissue. Ossicle composed of compact cortical bone (B) and healthy hematopoietic marrow (arrow). 60×.

injections. The labeled cartilage was transferred to the anterior chamber of the eye of another rat of the same inbred strain for a period of 3 weeks. There was no translocation of tritiated glycine from the matrix of the donor to the cytoplasm of the new cells of the host. Figure 77(*c*) is an autoradiograph of an area of osteogenesis in an excavation chamber in an isograft of articular cartilage.

By means of tritiated thymidine labeling experiments, one can see that the cells dissolve the capsule and surrounding matrix and undergo mitotic division. The first mitotic division produces connective tissue cells that look like mesenchymal cells. Six weeks after the operation an ossicle is formed (fig. 77(*d*)) and there is complete replacement of all of the donor tissue (fig. 77(*e*)).

The second, third, or fourth mitotic division—we do not know how many in 15 days—occurs in such a way that the capillary growing into the transplant interacts with the progeny of these germinal cells that have dedifferentiated or modulated, depending upon how one wants to look at it, and the interaction, or what Paul Weiss calls the *swarming* of these cells in two different locations, results in what appears to be a progenitor cell. We say this is a progenitor cell because its rate of uptake of tritiated thymidine is greater than that of any other cell in the area. It is by this criterion and by none other that we call it a progenitor cell. I am not even saying what kind, whether it is osteoblast, chondroblast, hematoblast, or something else.

PRITCHARD: You could call it a thymidine cell.

URIST: You could call it a thymidine-labeled connective tissue cell. The progenitor cells differentiate into osteoblasts. The interaction of the progeny of the cells from two sources produced bone. The progeny of one population of cells induced the progeny of the other to become osteoblasts and to make bone. The cells were not induced to make more cartilage in the new environment in which their progeny proliferated; instead, the product was new bone.

Figure 78 is a diagrammatic representation of the ordered sequence of events in the induction system for bone from articular cartilage. During the first 10 days after transplantation, the germinal cells of articular cartilage lyse the intercellular substance and divide mitotically to produce inducing cells. Swarming occurs between 10 and 15 days, and a large number of ingrowing perivascular inducible connective tissue cells of the host interact with a relatively small number of progeny of donor cells. Between 15 and 20 days, the donor tissue develops excavation chambers filled with sprouting capillaries and proliferating young connective tissue cells. Microscopically, the progeny of the donor and the host are identical, but functionally they are different insofar as some have been induced to differentiate or specialize as osteoblasts, chondroblasts, hematocytoblasts, or fibroblasts. Some

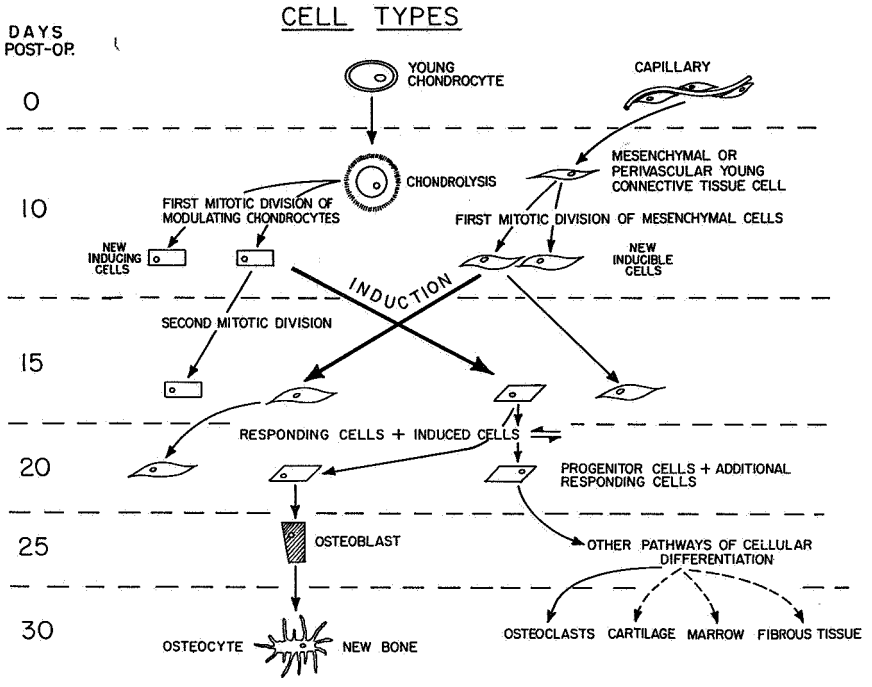

FIGURE 78. Ordered sequence of events in the induction system for bone from articular cartilage.

divide and produce more responding or undifferentiated connective tissue cells. Between 20 and 25 days, the induced cells differentiate into osteoblasts; between 25 and 30 days, the new bone containing osteocytes is deposited in one location while new marrow, fibrous tissue, and cartilage are deposited in another. Osteoclasts appeared only in association with bone tissue and with the onset of remodeling of the initial deposits.

The questions, Dr. Saxén, are these: What was transferred from one cell population to another? Was anything transferred? Was the inducer something that arose intercellularly as a result of this change in environment, or was some chemical inducer transferred from the donor to host progeny? Can you explain this reaction with the aid of your hypothesis?

SAXÉN: I am afraid, Dr. Urist, I cannot answer your questions. I pointed out that we do know that there are what I call epigenetic factors, but we do not know anything, or very little, about their chemical nature and their mode of action. For instance, you mentioned the anterior chamber of the eye. Kidney induction, as I showed, takes place under the very same conditions without its specific inducer. So

now there are people who are inclined to speak of "conditions" rather than of specific inducers.

So the only thing I can say is that these cells have been exposed to different environments. Whatever it is in these environments that causes this determination is not known.

I would like to know what Dr. Holtzer has to say about this; he is more familiar with these cells.

URIST: Dr. Holtzer, in the embryonic notochordal tissue-spinal cord induction system for cartilage, is there evidence that a substance is transferred from one cell to another?

HOLTZER: We have no evidence.

URIST: Is there evidence of a diffusible substance?

HOLTZER: Absolutely none.

URIST: In the experiments of Lash et al. (ref. 148), did cartilage induction occur from a substance that diffused across a Millipore membrane?

HOLTZER: There is an interaction. We never pretended that we had a molecule that we could name. We are asking a kind of question that is experimentally beyond our capacity, certainly my capacity right now, to analyze. That is why I prefer to refocus some of our questions in differentiation. We are all looking for little devils to send a cell in this direction, send a cell in that direction, and that's the end of it. On the contrary, there is excellent evidence in the case of bone that it is not that simple; that there are no demons that open trapdoors and let a message come through and that is the end of the interaction.

Therefore, why belabor something which people studying bone have demonstrated year after year? That is to say that there is continual interaction and that there are, in fact, alterations in the states of a cell. We define, by specifying the activity, what we mean by states of alteration.

The question you are asking, as far as I am concerned right now, is a rhetorical one because nobody has any evidence that an information-bearing molecule comes from one source, goes into another cell, and diverts that cell to another task.

PRITCHARD: In some setups, surely it does; you are a male and I am a male because at a certain stage in our development a certain hormone was produced by——

HOLTZER: Remember, I said "an information-bearing molecule" which changes the fate of a cell. By and large, hormones and other exogenous molecules accelerate or dampen an activity; they permit cells to express a capacity that was built into them by their previous history. To my knowledge there is no conclusive evidence that the known hormones establish that capacity. A hormone acts on a target cell. But the inductive influence, which initially guided the differentia-

tion of that target cell, was not that particular hormone. Hormones act on programed cells, not naive cells. Hormones select preset programs. The central problem of differentiation is the nature of the influences that establish the programs. At this point, with the possible exception of vitamin A, there is no clear-cut evidence of an "information-rich" molecule from one cell entering another and thereby altering the latter's fate.

PRITCHARD: A hormone is an information-bearing molecule.

HOLTZER: Does it go inside the second cell?

PRITCHARD: Yes.

HOLTZER: I am sorry; you might be right.

PRITCHARD: You see, once you label these things——

HOLTZER: I am sorry—I do not accept any of that work.

SAXÉN: I would like to add something to this matter of specific action. In quite another system, the developing central nervous system, it has been shown that factors such as CO_2 shock, a slight change in the pH of the environment, and things like that can trigger this chain of events. In these situations we certainly are not dealing with an information molecule, and that is one reason why I said I have my doubts about the specificity of this approach.

RAISZ: Dr. Urist, have you tested any changes in the microenvironment in the eye for the ability to induce bone; for example, calcium changes or something of that sort?

URIST: Some years ago in Dr. McLean's laboratory, Heinen (ref. 149) put rats on a phosphate-deficient diet, made them rachitic, cultured the bone, and transplanted the culture to the anterior chamber of the eye. The new bone was phosphate-deficient and vitamin D-deficient rachitic bone or osteoid tissue. The culture differentiated into bone, but the matrix did not calcify. Bone induction can take place even under conditions that are not conducive to calcification of the matrix.

RAISZ: That is what I was asking you: whether there were times that one could get cartilage or bone under some conditions of changing environment.

URIST: Yes; we will try to get to that question when we present a few more experimental observations.

We implanted a segment of HCl-decalcified lyophilized cortical bone into a pouch in the anterior abdominal wall, or the quadriceps muscle, and observed bone induction in excavation chambers inside the old matrix. Cartilage induction occurred inside old vascular channels.

FREMONT-SMITH: What was the origin of the decalcified bone? Also rabbit?

URIST: Yes, rabbit; we employed bone from the same or another individual.

FREMONT-SMITH: Is the effect not species specific?

URIST: No; it is not. We have also observed what Dr. Saxén calls heterogenous induction with implants of bovine and human matrix in rabbits (ref. 150), but the inflammatory reaction is high, the percent positive results low, and the yield of bone is very, very small.

I will now show some examples of bone induction by heterogenous, dead, decalcified bone matrix. Figure 79(*a*) is an implant of bovine decalcified bone matrix in the anterior abdominal wall of a rabbit. The implant is enclosed in an envelope of plasma cells, lymphocytes, reticulocytes, and foreign body giant cells. Figure 79(*b*) illustrates scanty deposits of new bone on the surfaces of an implant of dead, decalcified bovine matrix, inside the envelope of inflammatory tissue in the anterior abdominal wall of a rabbit, 12 weeks after the operation. Figure 79(*c*) shows the implant of bovine dead, decalcified bone matrix in the anterior abdominal wall of a rat, 12 weeks after the operation. The rat has a high propensity for formation of cartilage on the walls of vascular channels in the old matrix. These cells differentiate from histiocytes that wander into and repopulate nearly every crevice and space with new cells. In the process of cartilage induction, there is little or no resorption of the inducing surface.

It is necessary to emphasize, however, that while the inducing material is heterogenous in origin, in these systems both the inducing cells and the induced cells come from the host bed. For this reason, I refer to an article on bone formation by autoinduction (ref. 151).

We have implanted decalcified, lyophilized muscles, tendon, kidney, and other tissues into the anterior abdominal wall, and the results were negative over a period of 8 weeks. It is necessary to extend this experiment to 3 to 6 months; bone formation does not appear in injured tendons until after 3 months. Cartilage, however, is different; decalcified, lyophilized costal cartilage will induce bone formation with an incidence of over 60 percent positive results.

PRITCHARD: Dead cartilage and muscle will also show this, and there is a comparable delay before new bone appears.

URIST: Bone induction was influenced by the effects of the acid that was used to decalcify the matrix. Nitrous and nitric acids, which deaminate protein, prevent bone induction; HCl, EDTA, and formic-citric acid do not alter the matrix in a comparable way and do not inhibit bone induction. Nitric acid-decalcified matrix produces an extensive foreign body giant cell response.

Figure 80(*a*) illustrates a deposit of new bone on the walls of an excavation chamber in an implant of homogenous dead, decalcified bone matrix, 4 weeks after the operation in a rabbit. Figure 80(*b*) shows deposit of new bone and remnants of an implant of old dead, homogenous, decalcified bone matrix in a rabbit 8 weeks after the

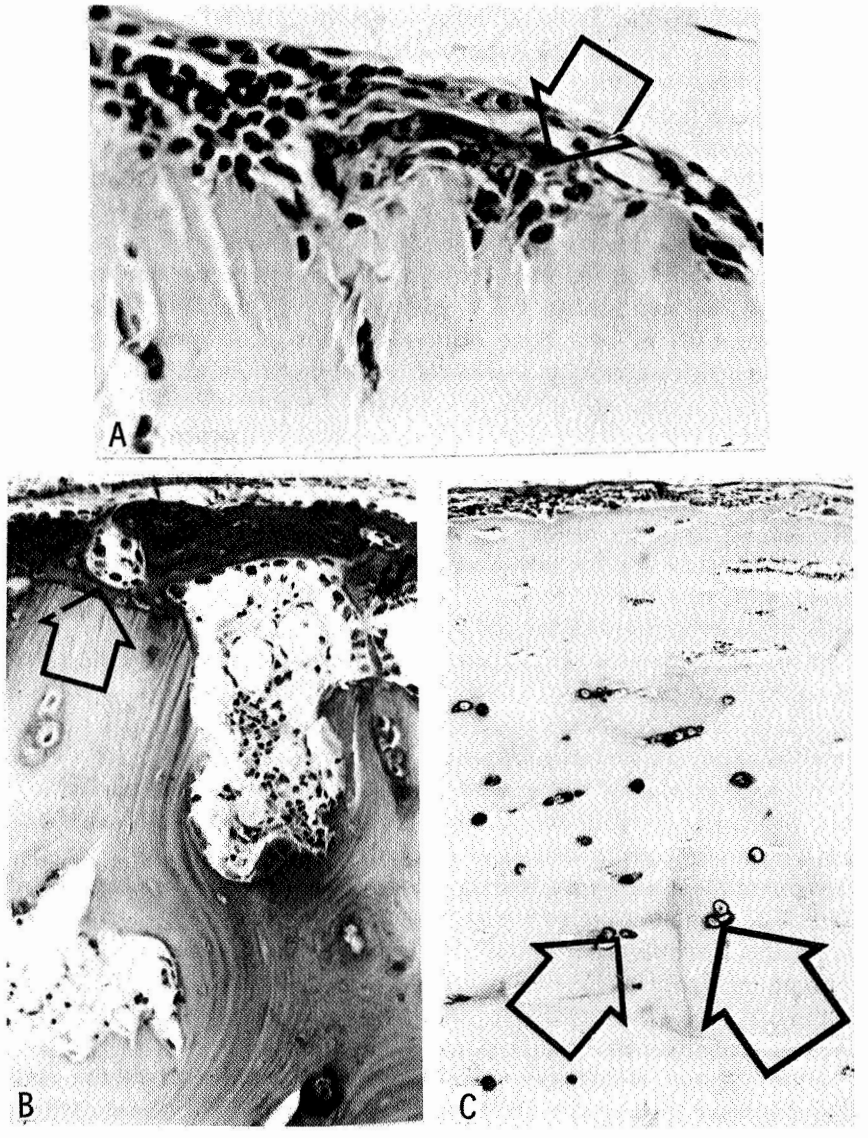

FIGURE 79. Photomicrographs of bovine decalcified bone matrix implants in the anterior abdominal wall of the following:

(*a*) *Rabbit*—the implant is enclosed in an envelope of plasma cells, lymphocytes, reticulocytes, and foreign-body giant cells (arrow).

(*b*) *Rabbit*—scanty deposits of new bone (arrow) on surface of implant inside envelope of inflammatory tissue.

(*c*) *Rat*—cartilage on walls of vascular channels in the old matrix (arrows).

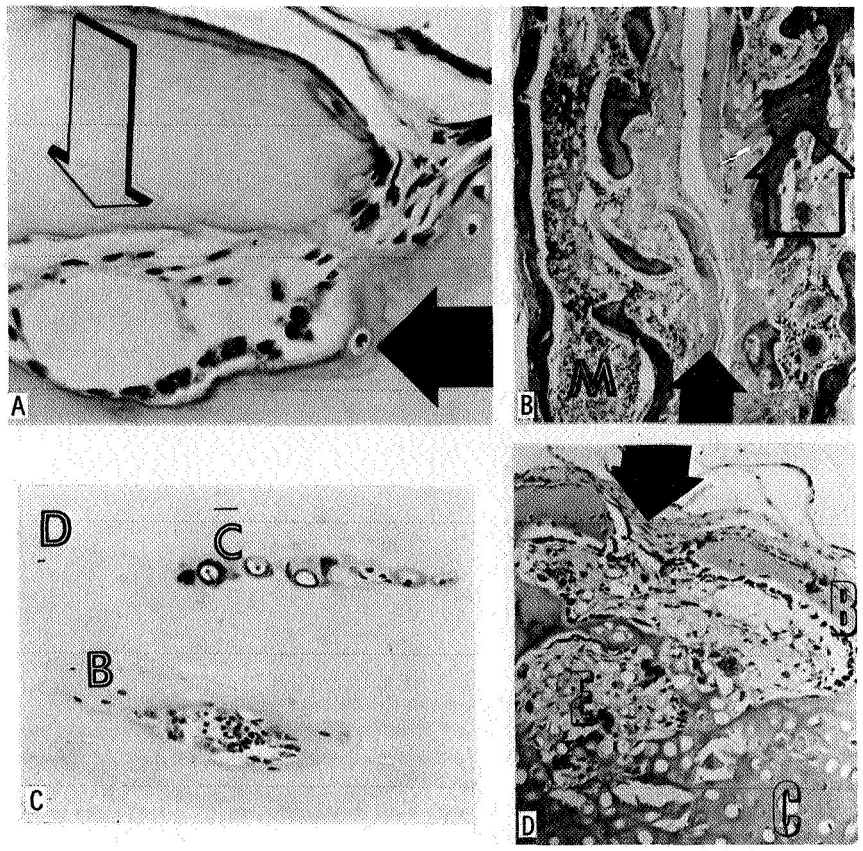

FIGURE 80. Photomicrographs of homogenous, dead, decalcified bone matrix implants in the anterior abdominal wall of a rabbit. HEA stain.

(*a*) New bone deposit on walls of excavation chamber; osteocyte (solid arrow) and empty lacuna (open arrow) in substance of the old matrix.

(*b*) New bone deposit (open arrow); remnants of old dead bone matrix (solid arrow); bone marrow (*M*).

(*c*) Decalcified, lyophilized bone matrix (*D*); chondrogenesis (*C*) in an old vascular channel; osteogenesis (*B*) in new excavation chamber.

(*d*) Decalcified, lyophilized costal cartilage; excavation chamber (*E*) filled with proliferating connective tissue in old acellular dead cartilage (*C*), deposit of new bone (*B*) and implant enclosed in a sheath of fibrous connective tissue (arrow).

operation. Figure 80(*c*) is an implant of HCl-decalcified, lyophilized bone matrix showing chondrogenesis in an old vascular channel and osteogenesis in a new excavation chamber. Figure 80(*d*) is a section of lyophilized, decalcified, homogenous costal cartilage, 8 weeks after implantation in the anterior abdominal wall of a young rabbit. The excavation chamber is filled with proliferating connective tissue

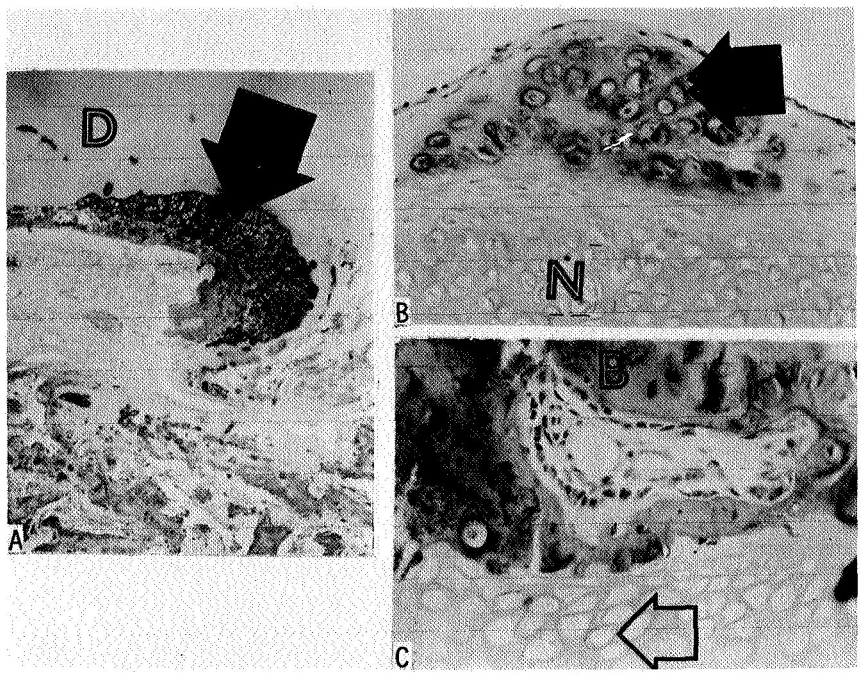

FIGURE 81. Photomicrographs of implants in the anterior abdominal wall.

(*a*) Island of new cartilage (arrow) on inner wall of decalcified homogenous bone matrix (*D*); new bone (bottom) and old dead matrix (top) surround area of chondrogenesis.

(*b*) Homogenous dead, decalcified, lyophilized nasal cartilage (*N*); induction of new cartilage formation (arrow).

(*c*) Homogenous dead, decalcified, lyophilized nasal cartilage (arrow); formation of excavation chamber and induction of new bone formation (*B*).

in old acellular dead cartilage, there is deposit of new bone, and the implant is enclosed in a sheath of fibrous connective tissue.

Figure 81(*a*) shows an island of formation of new cartilage on the inner wall of an implant of decalcified, homogenous bone matrix 6 weeks after the operation. Such masses of cartilage are later resorbed and replaced by new bone through typical endochondral ossification. New bone and old dead matrix surround the area of chondrogenesis. Figure 81(*b*) is an implant of homogenous, dead, decalcified, lyophilized nasal cartilage of a rat implanted in the anterior abdominal wall of a rat showing induction of new cartilage formation. Figure 81(*c*) is an implant of homogenous, dead, decalcified, lyophilized nasal cartilage showing formation of an excavation chamber and induction of new bone formation 6 weeks after the implant operation.

BÉLANGER: Were all of these made at the same time approximately?

URIST: The implants were excised at intervals of 4, 6, 8, and 12 weeks after the operation.

HOLTZER: Cells are invading the matrix. Can you say anything about them?

URIST: Yes; leukocytes, histiocytes, foreign body giant cells, sprouting capillaries, and perivascular connective tissue cells pass in review through the tissue in sequence at various times in various proportions, in various sites in the implant. The implant is at first unoccupied territory that gradually becomes repopulated with cells.

Figure 82(*a*) is homogenous, decalcified bone from a Belgian rabbit implanted in the anterior abdominal wall of a New Zealand rabbit without lyophilization, 4 weeks after the operation. Note the infiltration of inflammatory connective tissue cells and the dissolution of the bone matrix, but no new bone formation. Bone induction is retarded or suppressed by inflammation either from an immune response or from sepsis. Figure 82(*b*) is a roentgenograph of the anterior abdominal wall of a rabbit containing eight Millipore chambers. Four chambers contain transplants of fresh, autogenous, cancellous, viable bone tissue, and four contain 0.6 *N* HCl-decalcified dead bone matrix, 4 weeks after the operation. Bone tissue was formed inside, never outside, the membrane of the Millipore chamber containing viable bone tissue. There was no bone formation either inside or outside the chambers containing dead, decalcified bone matrix. When there was a defect in the Millipore membrane, the host cells were found in a stream growing into the chamber; these cells were able to gain contact with the dead matrix to set up an induction system for osteogenesis.

SAXÉN: The grafts are not rejected when you use a heterograft?

URIST: The plasma cell-reticulocyte reaction around the implant suggests that it is rejected. But it cannot be discarded. Instead, it is encapsulated in an envelope of inflammatory connective tissue cells. It is understandable that prolonged inflammation would inhibit cellular differentiation of cartilage and bone. Prolonged inflammation should induce differentiation of leukocytes and macrophages; cells specializing in phagocytosis, not in production of intercellular substances.

Except that cell specialization may begin in the aftermath of sterile inflammation at about 21 days, it is difficult to understand how chondrogenesis is induced in the blind end of an old vascular channel, or how osteogenesis is induced on the walls of an excavation chamber in decalcified matrix. In the case of cartilage, the surface of old matrix appears to be unaltered morphologically. On a molecular level, of course, it is possible to envisage a mechanism whereby the old matrix

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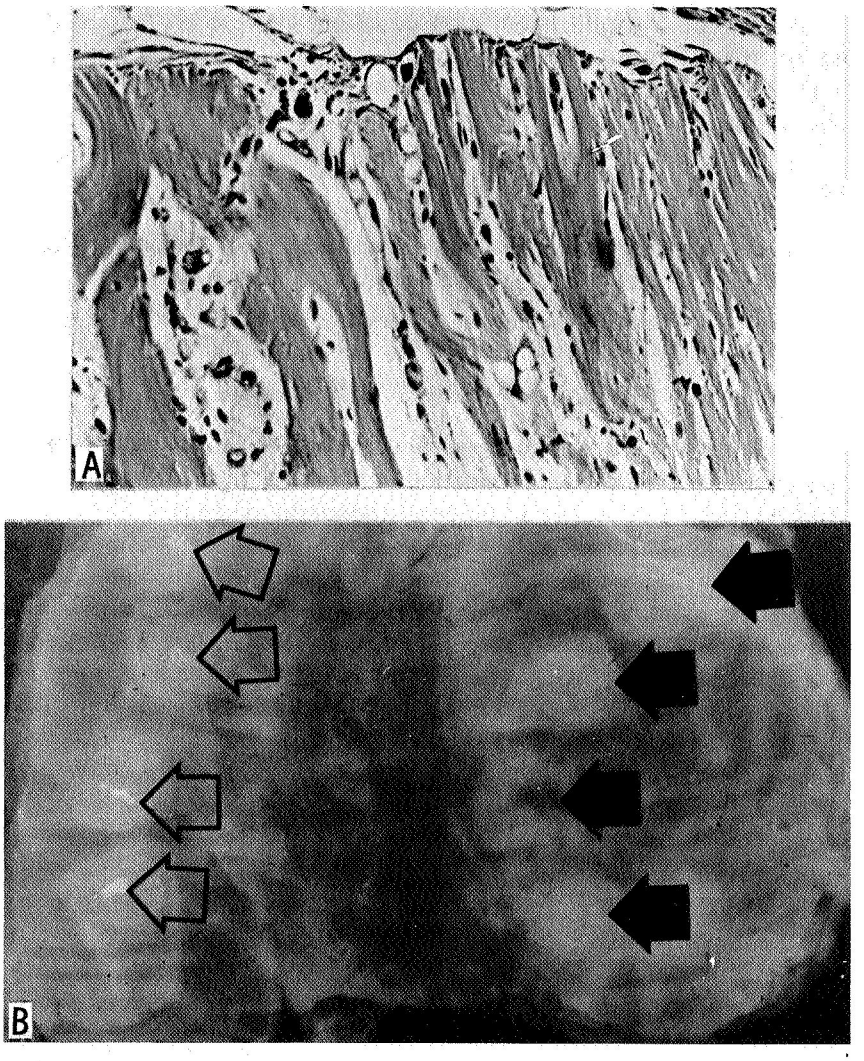

FIGURE 82. Implants in anterior abdominal wall of rabbit.
(*a*) Photomicrograph of inflammatory response from heterograft.
(*b*) Roentgenograph of abdominal wall with Millipore chambers *in situ;* fresh autogenous, cancellous, viable bone (open arrow); decalcified, dead bone matrix (solid arrow).

may transfer a template of protein structure onto the plasma membrane of young connective tissue cells; this can be transferred to the ribosomal membranes and then relayed to the regulator genes to produce cellular differentiation, either of a chondroblast or an osteoblast. These ideas, however, are conjectural and do not take into consideration many other factors in the host bed that are unspecific or unknown in nature.

PRITCHARD: I would like to show a figure of an implant of dead muscle that is being invaded by cells which turn into cartilage cells in an almost identical manner (fig. 83).

CURREY: Was this piece of muscle frozen?

PRITCHARD: No; it was fixed in acetone, but we are evidently dealing with a similar phenomenon.

URIST: The phenomenon is similar but not the same.

SAXÉN: Dr. Urist, do you have any information on the distance; that is, how far from the graft will new bone be formed?

URIST: The first row of cells in contact with the old decalcified matrix may differentiate into a row of osteoblasts, but we have seen the second row of cells produce bone without direct contact with the implant.

SAXÉN: How far from the graft is this new activity seen? If there is a transmission of something, there should be a maximal distance, which is quite well known in different induction systems.

URIST: The cells that grow in with a capillary sprout and produce an excavation chamber are the same cells that become osteoprogenitor cells, osteoblasts, and new bone. The distance across the excavation chambers produced by these cells is approximately 100 to 150 microns

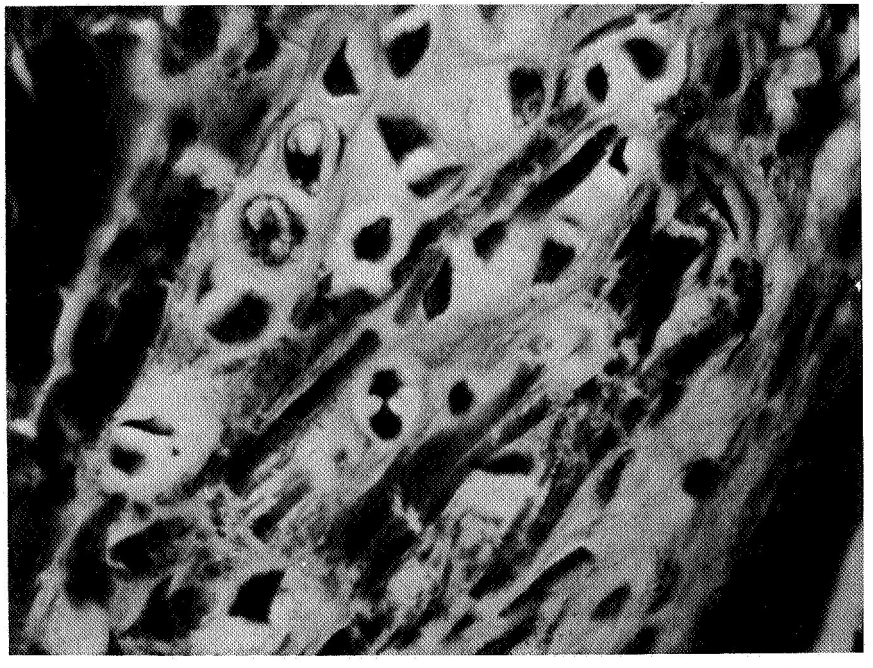

FIGURE 83. Photomicrograph showing rows of cartilage cells between fibers of acetone-fixed tongue muscle of the rabbit implanted beneath the kidney capsule. 500×.

in diameter. Does a chemical substance diffuse from matrix into the cytoplasm of the matrix-resorbing cells? Perhaps later I will present some experiments designed to potentiate, to degrade, or to inactivate the matrix that produced a bone induction system.

It should be noted that extraction or partial denaturation of the decalcified matrix with alcohol, strong salt solution, ether, acetone, and detergents did not retard bone induction. Autogenous bone matrix was not better than homogenous bone matrix for bone induction when implanted after devitalization, decalcification, denaturation, and extraction by these agents. Heating the implant to 100° C gelatinized the matrix and, like nitric acid and FDNB, prevented bone induction. Thus, although the structure of the protein of the bone matrix is an important factor, it is impossible to assume that something diffusible was transferred to ingrowing cells.

PRITCHARD: One can approach the problem in part by morphology. Whatever material is excavated, there is always a chance that the mesenchyme that goes in will make bone. It is a common phenomenon. The tunneling is done in the hard material; then the cells differentiate on the walls of the tunnels. Because these cells, sitting on the walls of the tunnel, are in direct contact with the bone, the distance is virtually nil. That was the question Dr. Saxén was asking.

URIST: The line of contact between the decalcified material and the cell might consist of one cell or one layer of cells; but the influence of the substance of the old bone matrix is upon a new population of cells, many of which do not appear to have direct contact with the implanted material. It is possible that a relay system is involved; the first layer of responding cells may become a layer of inducing cells, and layer by layer the inducer is transmitted from one cell to the next until the excavation chamber is filled with a laminated mass of bone except for a single blood vessel in the center.

PRITCHARD: Which one turned into the osteoblast, the one on the surface or the one in the middle?

URIST: Sometimes we see osteoblasts developing in an area separated from the old matrix by several layers of cells.

PRITCHARD: I have not seen this.

URIST: We do not see osteogenesis only in lines of contact with the old decalcified matrix; two or three layers of cells may be involved in the process in some areas of the resorbing implant.

I will try to present some experiments on matrix treated with blocking reagents and enzymes, designed to show whether or not something is transferred.

The question that we have before us is, What is the arrangement of the young connective tissue cells when a bone-induction system

begins in the interior of an implant of HCl-decalcified, lyophilized bone matrix, 21 days after the operation? Figure 84(*a*) is a section showing the old matrix, or inducing surface; the layer of young connective tissue cells, a mixture of inducing cells and reponding cells, in contact with the inducing surface; layers of proliferating fibrous connective tissue cells, or pool of new responding cells; and the mass of loose fibrous connective tissue and inflammatory cells.

Figure 84(*b*) is a higher magnification of the mixture of inducing and responding cells; the area indicated by (B) is the first layer in contact with the inducing surface, or old matrix. There is densely staining basophilic cytoplasm of some of the cells, resembling osteoblasts. Others with clear cytoplasm and dense nuclei resemble osteoprogenitor cells. Figure 84(*c*) shows connective tissue in the center of the implant of decalcified matrix in a rabbit given an arterial injection of india ink to label the macrophages. Deposits of new cartilage are seen in the old vascular channels. A higher magnification

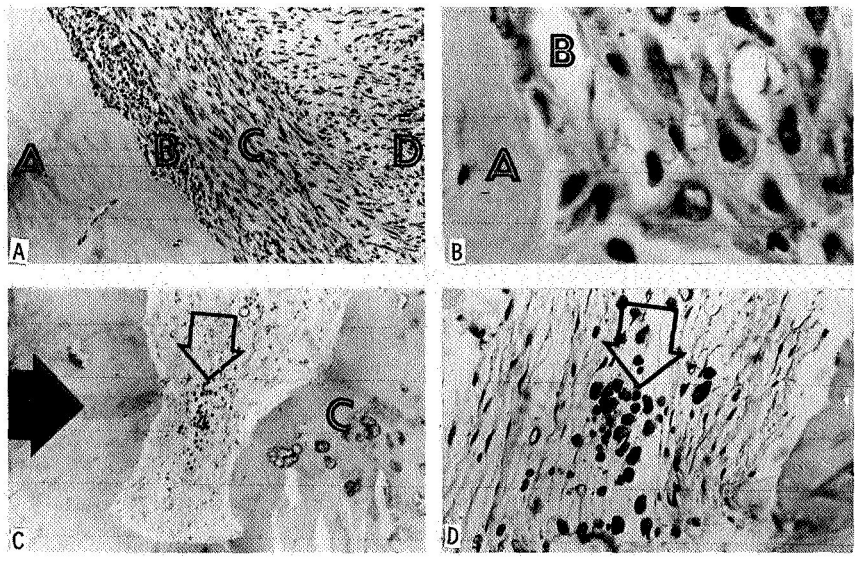

FIGURE 84. Photomicrographs of sections of HCl-decalcified, lyophilized bone matrix implants 21 days after the operation.

(*a*) Old matrix (*A*), layer of young connective tissue cells (*B*), layers of proliferating fibrous connective tissue cells (*C*), and loose fibrous connective tissue and inflammatory cells (*D*).

(*b*) Old matrix (*A*); first layer in contact with inducing surface (*B*).

(*c*) Center of decalcified matrix (solid arrow) after injection of india ink to label macrophages (open arrow). Note deposits of new cartilage (*C*) in old vascular channels.

(*d*) Location of macrophages filled with phagocytosed particles of india ink; none was found in macrophages in contact with old decalcified matrix.

of this area is shown in figure 84(*d*) to demonstrate the location of macrophages filled with phagocytosed particles of india ink; none was found in macrophages in contact with the old decalcified bone matrix.

In summary, the observations on early stages of bone induction suggest that the young connective tissue cells in contact with the bone matrix are several layers in thickness. During the period from 19 to 23 days after the operation, the young proliferating cells may move on and off the inducing surface, interact and swarm. At approximately 24 days a layer of osteoblasts appears, and bone is formed on the inducing surface of old matrix. After that, the layer of new bone may become the inducing surface, and the next layer of osteoblast may become the inducing cells, and so on.

To determine the sequence of events and the mitotic activity of the layer of inducing cells in contact with bone matrix, the host was injected with ^{3}H-thymidine and the implant excised at 1 and 5 days later during the 19- to 23-day period of bone induction. Autoradiographs demonstrated that the cells with the highest percentage of labeled nuclei were located along the inducing surface of old matrix and near areas of osteogenesis. The cells with the lowest percentage of labeled nuclei were located in areas of resorption and phagocytosis and some distance away from areas of osteogenesis. The percentage of labeled cells was also low in areas of chondrogenesis, possibly because the rate of mitosis of the cartilage cells is relatively low compared with that of osteoprogenitor cells.